GENERAL SESSION I

Discussion Leaders:

DR. MARSHALL R. URIST
DR. FRANKLIN C. MCLEAN

MCLEAN: To avoid any misunderstanding, I would like to expand on the discussion of the previous session concerning the effect of parathyroid extract in the rat. The following figures illustrate sections of bones taken from rats that received 1000 units of parathyroid extract. This is old stuff, but it is so old I think it has been largely forgotten, so I can bring it to life again.

Figure 108 shows a section of a tibia from a 6-week-old normal control rat with about the normal amount of primary spongiosa and very active osteoblastic activity. Figure 109 shows the tibia 9 hours after PTE administration. At greater magnification, pyknotic nuclei of osteocytes may be seen and also the cluster of osteoclasts around the spicules of bone that are undergoing resorption. Figure 110 is a photomicrograph 24 hours after PTE administration, and the primary spongiosa has virtually collapsed and is amputated from the secondary spongiosa; there has been very widespread and very active resorption in the area of the primary spongiosa.

Figure 111 shows the tibia 4 days after PTE administration; note the amount of reconstruction and new bone. All of the bone in the spongiosa is new bone that was formed after the initial phase of resorption, which almost destroyed the spongiosa.

Figure 112 is a higher magnification of figure 111, and there is construction of new bone with very active osteoblasts throughout. This is what used to be called hyperostosis, and the point that I made previously was that this reaction has been observed only in the rat. I also made the point that by controlling the dosage and the timing, either an osteoblastic or an osteoclastic reaction may be produced. With smaller doses of parathyroid extract—and remember, we are dealing with Lilly's parathyroid extract—it is possible to arrive at this stage of hyperostosis without ever going through the stage of resorption and osteoblast formation.

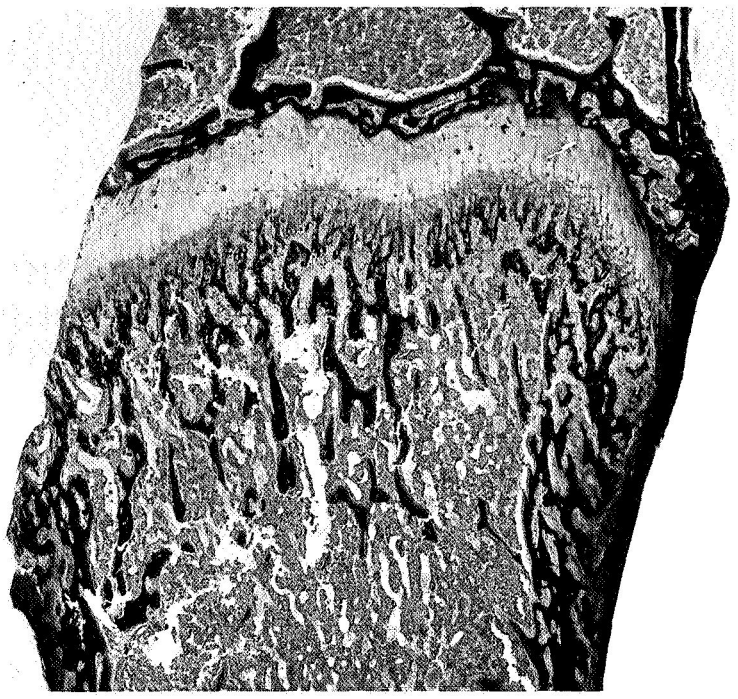

FIGURE 108. Photomicrograph of a longitudinal section of a tibia from a normal rat, approximately 6 weeks old. Zenker-formol fixation; HEA stain. 16×.

RAISZ: Do those small doses cause hypercalcemia?

MCLEAN: With doses smaller than those that will produce hypercalcemia, one can produce hyperostosis, or the growth of new bone. Again, I emphasize that this is in the rat; since the rat does react this way, one can account for Dr. Young's ability to produce at will, by proper timing and proper dosage, osteoclasts or osteoblasts from osteoprogenitor cells.

NICHOLS: Were these repeated doses, Dr. McLean?

MCLEAN: To achieve the osteoblastic reaction without the osteoclastic reaction, small doses of around 50 units must be given daily over a period of 3, 4, or 5 days. This will produce a lot of new bone, but there will be no stage of osteoclastic resorption.

NICHOLS: I ask because we conducted a somewhat similar experiment and found that there is indeed a period lasting a day or two when there is a fall of collagen biosynthesis from proline. Later, this effect is reversed and an increase can be shown (ref. 152). Our data are very similar to yours in that respect. We had, however, some degree of hypercalcemia in these animals. Also we found an increase by the

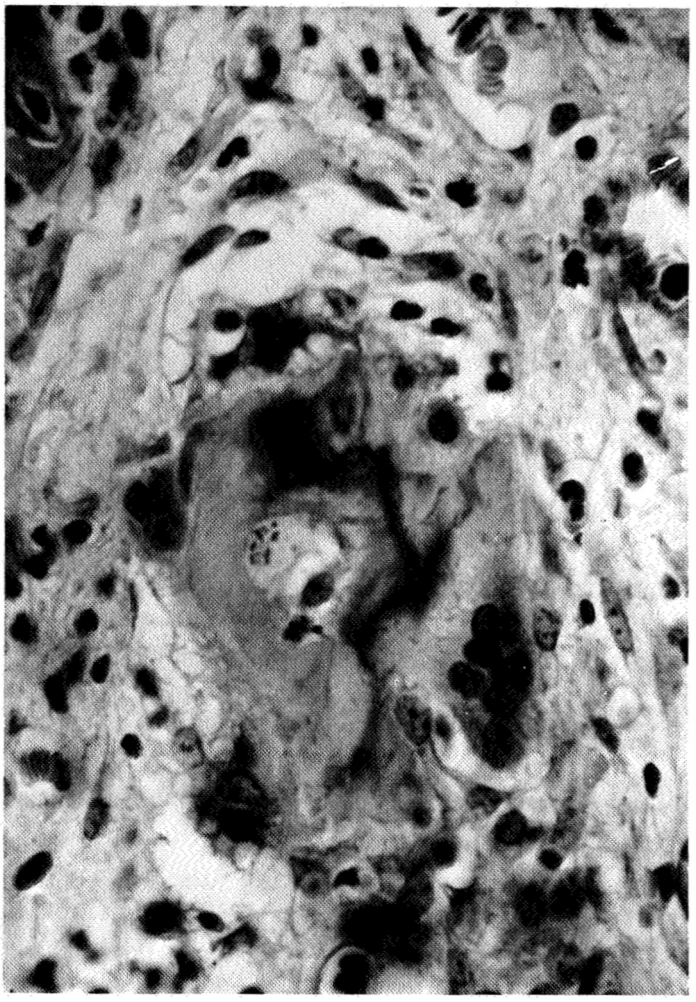

FIGURE 109. Photomicrograph of a section of a tibia from a normal rat 9 hours after administration of 1000 units of parathyroid extract. Note the pyknotic nuclei of osteocytes and the cluster of osteoclasts around the spicules of bone that are undergoing resorption. Zenker-formol fixation; HEA stain. 800×.

tissue in lactate production which starts right away at day 1 and gradually builds up to its final level.

MCLEAN: In the intact rat, if the dose is small enough, osteoblastic activity will appear in new bone formation without any stage of resorption.

BÉLANGER: Dr. McLean, do you know whether this new bone which was formed under these conditions is normally mineralized or is it undermineralized bone?

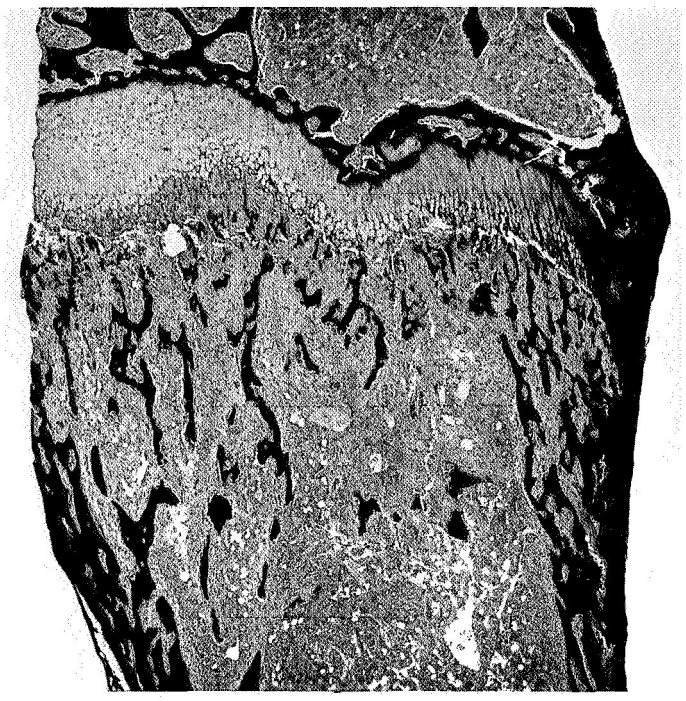

FIGURE 110. Photomicrograph of a longitudinal section of a tibia from a normal rat 24 hours after administration of 1000 units of parathyroid extract. The primary spongiosa has collapsed and is amputated from the secondary spongiosa. Zenker-formol fixation; HEA stain. 16×.

MCLEAN: In the stage that is shown in figure 112, it is undermineralized. It has not had time to catch up with the very rapid formation of bone.

BÉLANGER: You would not call this osteoid?

MCLEAN: No; it is not osteoid. It is partly mineralized.

PRITCHARD: Can anyone tell me exactly what calcitonin does to a bone-cell population?

COPP: Aliapoulios and Munson (ref. 174) have suggested that you may get stimulation of bone formation as well as inhibition of resorption.

PRITCHARD: Has the actual cell population been studied histologically?

COPP: No.

NICHOLS: We have some data on the response of the tissue, again in terms of the proline incorporation into collagen. This is increased, although it is not the first response seen after administration of the hormone. I cannot tell you whether this happens in parathyroidectomized animals.

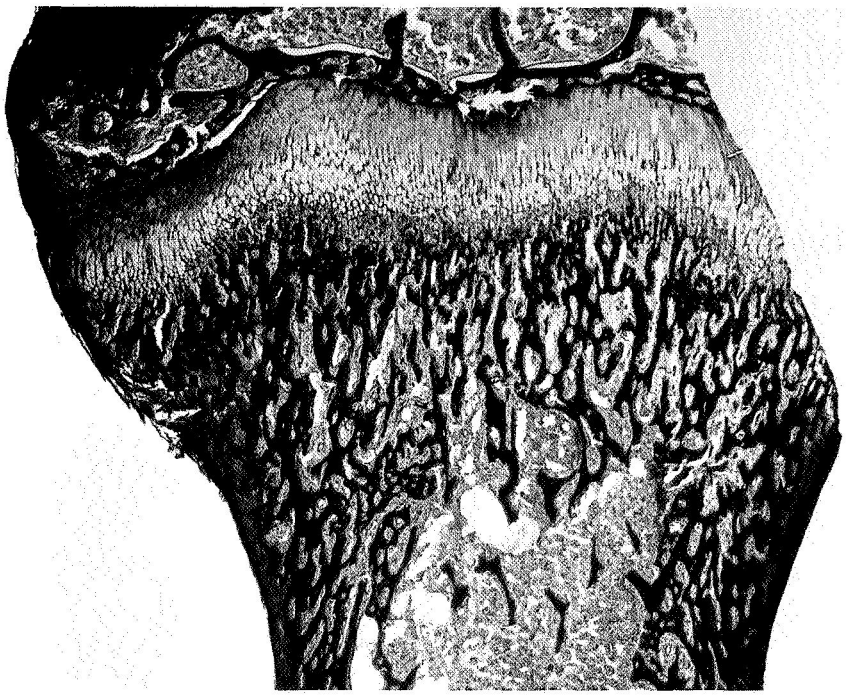

FIGURE 111. Photomicrograph of a longitudinal section of a tibia from a normal rat 4 days after administration of 1000 units of parathyroid extract. Note the amount of reconstruction and new bone. Zenker-formol fixation; HEA stain. 16×.

TALMAGE: With reference to different effects produced by varying the dose of parathyroid hormone, I was with Professor Gaillard a couple of years ago and we were doing organ culture—I must be very careful to talk about organ culture instead of tissue culture—of radii taken from embryonic mice. It was observed that if a radius was cultured from 18-day embryos with 0.01 unit of PTE for a 24-hour period, what looked like osteolysis occurred. There were almost no osteoclasts formed. However, in bone already formed in the embryo, the lacunae became larger, indicative of resorption. If the other radius taken from the same embryo was cultured with 0.1 unit, in just a matter of 8 hours large osteoclasts were formed. This, then, is a differentiation because of the dose. I believe the difference is based on the fact that there are two different types of action of parathyroid hormone in bone. One effect of the hormone is in rearranging or reorienting the DNA–RNA relationship. Normally this effect is only seen in mesenchyme cells, but if a large dose is administered not only the mesenchyme cells are affected, stimulating the production of osteoclasts, but also the osteoblasts are affected. At these high doses the

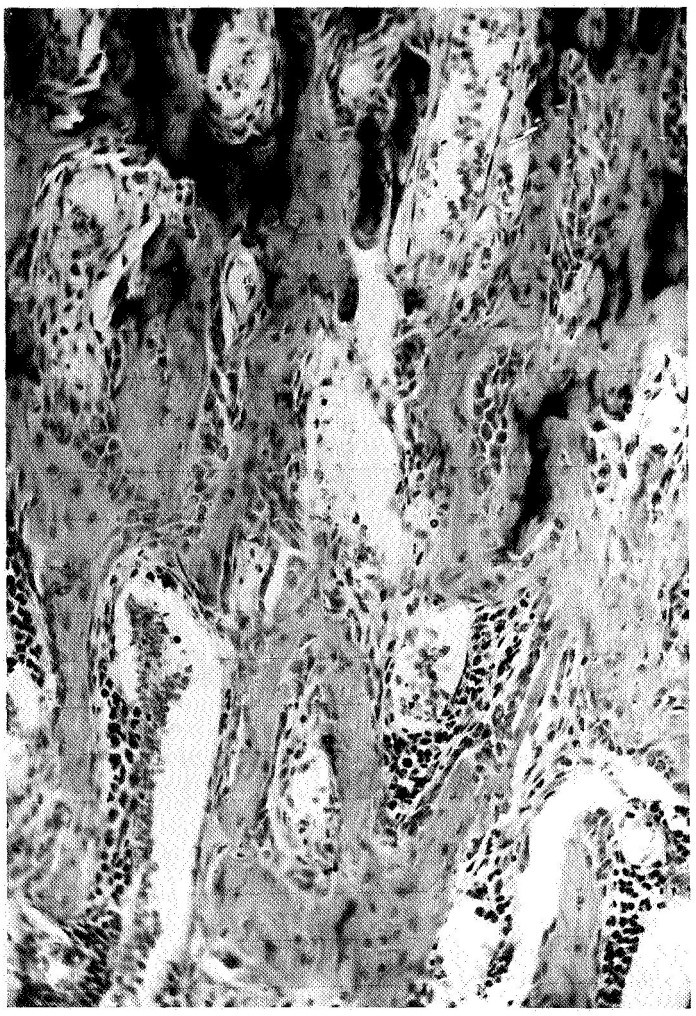

FIGURE 112. Higher magnification of figure 111 showing construction of new bone with active osteoblasts. 200×.

hormone affects osteoblasts by suppressing their ability to synthesize protein, and reorients them to something, either back to mesenchyme cells or—well, they just change.

I think this is a matter of the sensitivity of these cells, in a particular animal species, to the dose level for this particular function of this particular hormone.

MCLEAN: This is what I was trying to emphasize, that what we are seeing in the rat is something that we have never been able to produce in any other laboratory animal. The rat has its peculiar response,

and we have never seen this hyperostosis, particularly the osteoblastic reaction with the formation of a lot of new bone in the metaphysis, in any other species.

TALMAGE: I feel that all the work that Dr. Nichols does on the effect of parathyroid hormone on inhibition of protein synthesis is an example of this type of effect. In the rat, parathyroid hormone will inhibit a mechanism in osteoblasts, probably biochemically the same type of mechanism which exists in the mesenchyme cells. However, it takes a higher dose to produce such effects in osteoblasts.

PECK: Occasionally in the clinical syndrome of primary hyperthyroidism, we do see patients who have increased bone production locally. It is not really a hyperostosis, but local increases in bone production within the confines of the bone, presenting an osteosclerosis.

MCLEAN: That is why this condition was long ago termed "osteitis fibrosa"—because in chronic cases of hyperparathyroidism a fibrous reaction with overproduction of bone and fibrous tissue has been reported.

PECK: It is bone, primarily.

MCLEAN: It is bone, but surrounding the bone there are a lot of cells that look like fibroblasts. I would like to demonstrate again the reversal that occurs spontaneously in the bird (ref. 175). Figure 113 shows sections of bones from a pigeon. Figure 113(*a*) is the osteoblastic stage when the pigeon is forming medullary bone, and one sees almost a pure culture of osteoblasts with every sign of a great deal of activity. Suddenly, when the egg starts moving down the oviduct and acquiring a shell, the whole picture changes and the spicule of bone (fig. 113(*b*)), instead of being surrounded by osteoblasts, is surrounded by osteoclasts and is undergoing rapid resorption. This lasts, in the pigeon, for about 24 hours, and during the time the egg is being coated with the shell.

We are certain that this is not a parathyroid phenomenon. We tried to reproduce this picture with parathyroid extract in birds and did not succeed. But something triggers a reversal so that in place of the osteoblasts there are only osteoclasts, and resorption occurs at a rapid rate.

NICHOLS: May I say something more about this biphasic parathyroid effect? Dr. Flanagan and I published a paper not long ago in which we tried to relate the changes in bone-cell metabolism to the amount of hormone that was being poured out, or if you like, the frequency with which the total amount of hormone was excessive (ref. 152). Some patients showed an inhibition of proline incorporation into collagen, while others showed a considerable stimulation. This seemed to be related to how active the disease was.

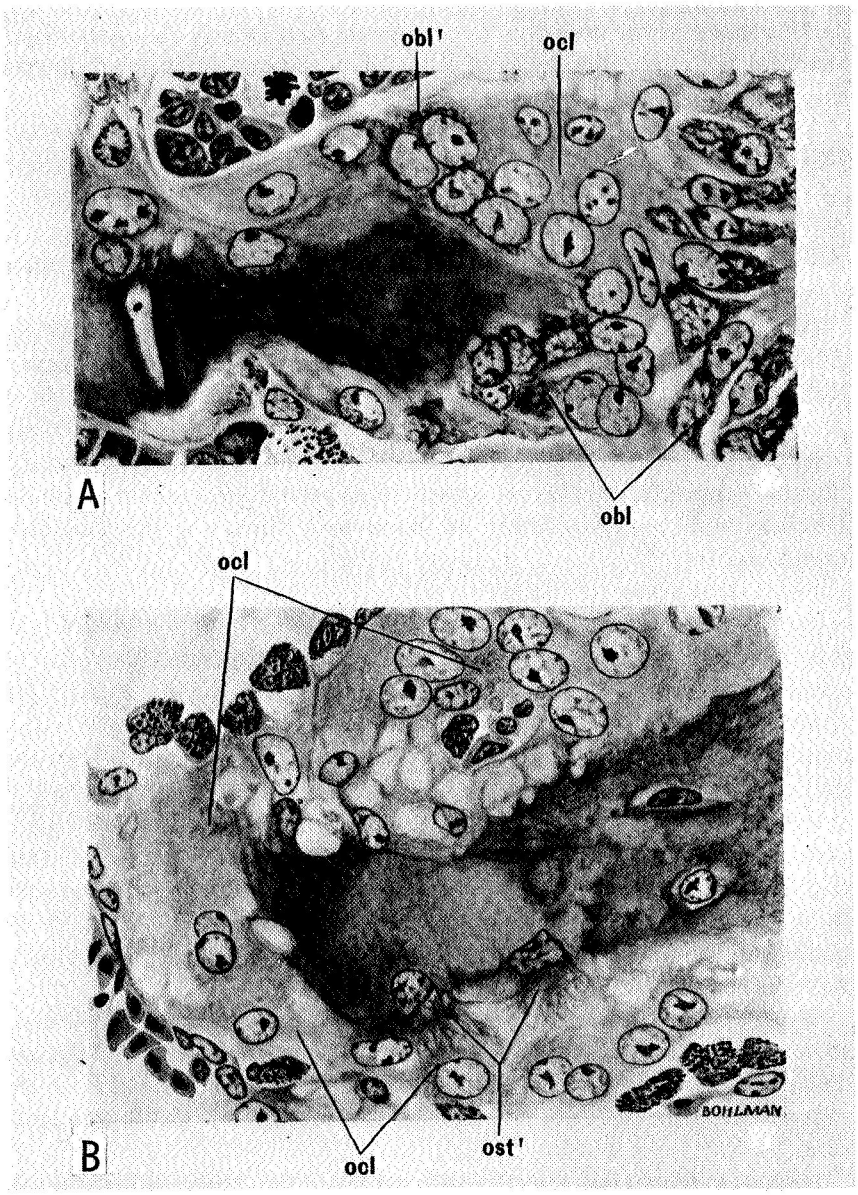

FIGURE 113. Photomicrographs of a section of bone marrow of a pigeon killed 25 hours after laying the first egg. (*a*) A stage in the transformation of an osteoclast (ocl) into osteoblasts (obl) through the accumulation of basophilic cytoplasm (obl') around the nuclei of the osteoclasts before the separation into individual cells. (*b*) During the process of breakdown of bone, liberated osteocytes (ost') fuse with an osteoclast (ocl). Zenker-formol fixation; HEA stain. 425×. [From ref. 175; reprinted by permission of the publisher.]

Those who had flagrant disease all showed stimulation of new bone formation. We suggested that these were the patients in whom the destructive process was tremendously accelerated, and we thought we were seeing the end result of the well-known link between resorption and accretion, which seems to be present in most animal species. On the other hand, for the patients in whom there was inhibition of collagen synthesis, the diagnosis seemed to be exceedingly difficult to make. Hypercalcemia and hypercalciuria were only occasionally present and evidence of bone disease was totally lacking. Nevertheless, these people had adenomas and were apparently cured by their surgical removal.

ARNAUD: I think that we are dealing with a terribly complex situation when we try to interpret the manifestations of parathyroid hormone excess. The responses of the intact animal to the administration of parathyroid hormone are almost certainly not related exclusively to parathyroid-hormone action. There is little question that the hypercalcemia produced alters the production and secretion of other hormones as well as changing the distribution of anions and cations across biologic membranes. Thyrocalcitonin is in this regard a very important hormone.

Now, the mode of action of thyrocalcitonin is unknown, but I think we are all agreed that it acts on bone. In the studies that have been shown, we have not been told that the animals were thyroidectomized. If they were not, we must assume that they all had high circulating levels of thyrocalcitonin.

Also, much of the work that has been done *in vitro* has been with commercial parathyroid extract. It should be noted that very likely there are contaminating materials in this extract that have a profound influence on biologic systems. Any time an effect has been demonstrated *in vitro* with parathyroid extract, the same effect should be expected when the highly purified hormone is used. If it is not demonstrated, the possibility must be considered that a contaminant is causing the effect.

YOUNG: Some of us have used parathyroid extract for purposes other than elucidation of the effects of endogenous parathyroid hormone in man. We are well aware that commercially available beef parathyroid extract contains an appreciable amount of nonhormone contamination. The point of using it, and the point of using the rats, which respond very overtly to the extract, was to demonstrate that the cells of bone are capable of assuming different specializations under different environmental conditions. As I saw it, this was the significance of the work I presented here. The observation that the bone cell can assume different functional states to meet different circum-

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stances can be demonstrated in many ways and, in fact, was supported several years ago by Dr. Bloom and Dr. McLean, using different systems, including the egg-laying birds which we saw here (refs. 135, 175, and 176).

It can be shown in a fracture site, or *in vitro* by changing the culture conditions, or in the induction experiments of Dr. Urist, and so on. I would just like to emphasize that I do not think there is any disagreement between Dr. McLean and myself on the ability of these bone cells to change their specialization (as some of you might have been led to believe). The demonstrated ability of the bone cell to adapt itself to changed conditions is perhaps one of the more important findings to grow out of this work.

LLOYD: Just for the record, perhaps I ought to say that at the 1967 British Bone & Tooth Society meeting on calcitonin, Dr. Gaillard presented some work on embryonic mouse radius explants. He spoke on the subject of growth and found an increase in the number of osteoclasts using parathyroid extract. By adding thyrocalcitonin, he was able to negate this effect and produce a much larger number of osteoblasts.

NICHOLS: I think Dr. Arnaud's point needs to be reemphasized, which indeed your report does, because if one removes various glands from rats one can find a whole hatful of different changes in bone-cell metabolism. For example, a pituitary must be present for normal metabolic activity; the same kind of change one obtains with hypophysectomy can be reproduced by removing the thyroid; administration of TSH to a hypophysectomized animal restores collagen biosynthesis nearly to normal. Therefore, we must seriously consider using more or less completely ablated animals if we are going to define more precisely the various primary effects of these hormones on the system.

COPP: The point Dr. Arnaud is making is that matters are complicated when a hormone is given which produces changes in ionic concentrations of calcium and phosphate in blood. This may affect the release of other hormones or may have a direct effect on bone and kidney.

ARNAUD: I do not think that we should be such purists that we exclude all of the actions, the actually observed actions, of parathyroid hormone in nonablated animals, but I do think we ought to hold some things in reserve. I wonder about the osteosclerotic type of response that is seen in the intact animal after parathyroid hormone has been injected. I think this may be related to other hormones.

TALMAGE: Just a word in opposition to this. Such a response can be produced without ever raising the plasma calcium concentration. In our experimental procedures we were able to get what I once glibly

called a "flower garden of osteoblasts" without ever pushing the plasma calcium concentration above 10 mg/100 ml.

ARNAUD: I am not talking about the classic osteoclastic response. I am a believer.

TALMAGE: I am very happy to hear you say this because for 10 years I have insisted that the best way to study parathyroid function is not to use exogenous hormone, but to cause the animal to increase its own hormone production.

ARNAUD: I think that this may be the best way of going about it. On the other hand, it is not the easiest way, and we are not all going to sit down and do peritoneal lavage.

RAISZ: There is one further proviso with regard to tissue-culture studies. Most tissue cultures employ serum in the medium, and serum is a very complex material. We have attempted to eliminate serum in order to eliminate factors that complicate our studies as well as experiments in the living animal. The hormones can be removed by using serum from thyroparathyroidectomized animals. Such serum will support bone resorption in tissue culture. When you try to get rid of the serum, the parathyroid response to bone resorption disappears. Adding back various components of serum has indicated that it is the albumin fraction of serum which supports the parathyroid response in tissue culture (ref. 177, work done in collaboration with Dr. Paula H. Stern, Department of Pharmacology, University of Michigan School of Medicine). Whether or not the factor or factors involved have any importance *in vivo* certainly deserves further study.

FREMONT-SMITH: I think we have to be extremely careful at all times, but nobody suggested that tissue culture was the answer to all problems. It has been suggested that it is the answer to no problems, and I think you have brought out very nicely the fact that you made a discovery by working with tissue culture of something which you did not know was there.

One of our problems is that we are constantly trying to take the animal apart to see how the parts work, and every time we do this we find it is much more complicated than we thought it was and we try to put it back together again. This is a constant interaction process. It is interesting that when you get a group of people together from different disciplines, each one rejects the data from the other disciplines because it is much too complicated and awfully messy. So I am just bringing out the fact that up until the time I mentioned tissue culture it had not been mentioned, and it seems to me it is a very important thing to mention with all of the dilemmas it is going to produce. It will produce another whole series of dilemmas, and they may throw more light on how large our area of ignorance is.

TALMAGE: I have been instructed by Dr. Bauer to apologize for speaking so strongly against tissue culture in the last session. I really have great faith in tissue-culture techniques, and I apologize.

URIST: This seems like the time for Dr. Copp to make some concluding remarks, and the time to extract some concluding statements from the discussants of the subject of the first session, "Homeostasis of Calcium."

NICHOLS: While Dr. Copp is preparing this material may I just tell Dr. Arnaud that as far as the sclerotic reaction described or the stimulation by parathyroid hormone is concerned, it is indeed true that one must have both a pituitary and/or a thyroid. Probably it is the thyroid, because TSH-treated, hypophysectomized animals do seem to have some response.

ARNAUD: Thank you.

COPP: In drawing the discussion of calcium homeostasis to a close, I would like to speak briefly of evidence for possible parathyroid involvement in control of hypercalcemia and then discuss some studies on dietary factors in homeostasis.

In most species, the parathyroids are embedded in thyroid or are so closely associated with it that it is difficult to separate them. In many of the earlier experiments, parathyroidectomy was performed by removing the thyroid as well, although this is not always mentioned in the paper. Now that the importance of the thyroid in calcium homeostasis is recognized, it is important to make a clear distinction between the parathyroidectomized and thyroparathyroidectomized state. In the dog, it is impossible to perfuse the parathyroids independently of the thyroid, and for this reason, our original experiments on gland perfusion did not distinguish between a thyroid or parathyroid origin for calcitonin. However, in the sheep and pig, the superior parathyroid which makes up 90 to 95 percent of the total parathyroid tissue, is quite distinct with a separate blood supply, so that the two glands can be perfused independently (ref. 178). As shown in figure 114, high calcium perfusion of the thyroid in sheep caused little change in plasma calcium concentration at 1 hour, but there was a slow and steady fall thereafter. The response to high calcium perfusion of the parathyroid was a prompt fall in systemic plasma calcium. This observation has been confirmed by Care et al. (ref. 179), who also observed a hypocalcemic response to injections of certain parathyroid extracts. MacIntyre et al. (ref. 180) observed, in dogs, that the fall in plasma calcium concentration was greater when thyroid and parathyroids were perfused with high calcium blood than when only the thyroid was involved. However, the evidence that parathyroid calcitonin may play a role in controlling hypercalcemia in some species is still tenuous,

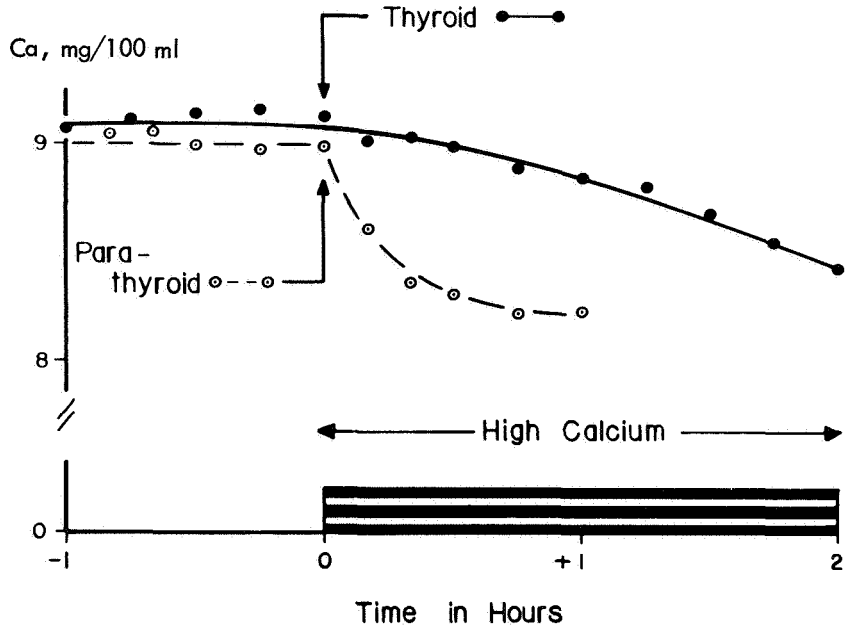


FIGURE 114. Changes in plasma calcium associated with high calcium perfusion of the thyroid or the superior parathyroid in 18 anesthetized sheep.

particularly when compared with the very substantial evidence for an important function for thyroid calcitonin.

I would now like to turn to our studies on the effect of dietary calcium and phosphate on calcium homeostasis in young rats. These experiments were carried out by Miss Anne Kuczerpa, who has developed an excellent ultramicro method for calcium, based on color development with glyoxal bis(2-hydroxyanil). Each determination requires only 0.02-milliliter plasma, so that as many as 10 samples of tail blood may be analyzed in the same animal.

The composition of the synthetic diet is given in table XXIII. It uses purified beef fibrin as the protein source and is essentially free from calcium and phosphate unless supplemented as indicated. It also contains approximately seven times the minimum requirement of vitamin D.

One of the real problems with a synthetic diet is to persuade the rats to eat and, as shown in figure 115, we go to considerable lengths to make it attractive. The most important feature is the rum flavor.

With the low phosphate diet, there is a fall in plasma phosphate and a rise in plasma calcium concentrations within 24 hours. This is apparent even when calcium is also absent from the diet, as shown in

TABLE XXIII

COMPOSITION OF THE CALCIUM- AND PHOSPHATE-FREE DIET (PER KG)

Basic ingredients:	*g*
Beef blood fibrin	350
Corn starch	380
Sucrose	175
Alphacel	40
Vitamin A oil	2
Vitamin D oil	2
Vitamin mix	25
Salt mix (varies)	17
Salts:	*g*
Sodium bicarbonate	14.6
Potassium carbonate	1.39
Ferric citrate	.5
Magnesium chloride	4.2
Potassium chloride	.95
	mg
Cupric sulfate	75
Manganous chloride	143
Potassium iodide	.22
Cobaltous chloride	5
Zinc chloride	2
Vitamins:	*mg*
Thiamine HCl	10
Riboflavin	10
Pyridoxine HCl	5
PABA	5
Vitamins—*Continued*	
Folic acid	10
Niacin	50
Ca pantothenate	50
Inositol	200
	g
Ascorbic acid	1.0
Choline	1.8
	IU
Vitamin A	20 000
Vitamin D	8 000
Supplements: *g*:	
A. Normal Ca (0.40%) Low P (0.02%):	
Add:	
Calcium carbonate	10
Sodium bicarbonate	14.6
B. Normal P (0.40%) Low Ca (0.01%):	
Add:	
Sodium bicarbonate	14.6
$NaH_2PO_4 \cdot H_2O$	8.5
$NaHPO_4$	8.5

figure 116, although the effect is not so great. This would suggest that it is the low phosphate concentration in blood rather than the calcium in the gut that is responsible. There is some indication that the low concentration of blood phosphate inhibits bone formation and increases osteolysis, thus raising the calcium concentration even when none is available from the gut (ref. 181). The effect of the phosphate-deficient diet was also observed in thyroparathyroidectomized rats. As shown in figure 116, within 24 hours of feeding a low-calcium, low-phosphate diet, the plasma calcium concentration had returned to normal, and within the next 2 days, there was a slight hypercalcemia. Certainly, it is rather surprising to obtain hypercalcemia in a young parathyroidectomized rat which has been fed a calcium-deficient diet. It also indicates that phosphate may have an important role in calcium homeostasis, even in the absence of the thyroid and parathyroid glands. We found that the presence or absence of the thyroid made no difference in the degree of hypercalcemia in these animals, and this agrees with our

FIGURE 115. Typical procedure for feeding rats the synthetic diet.

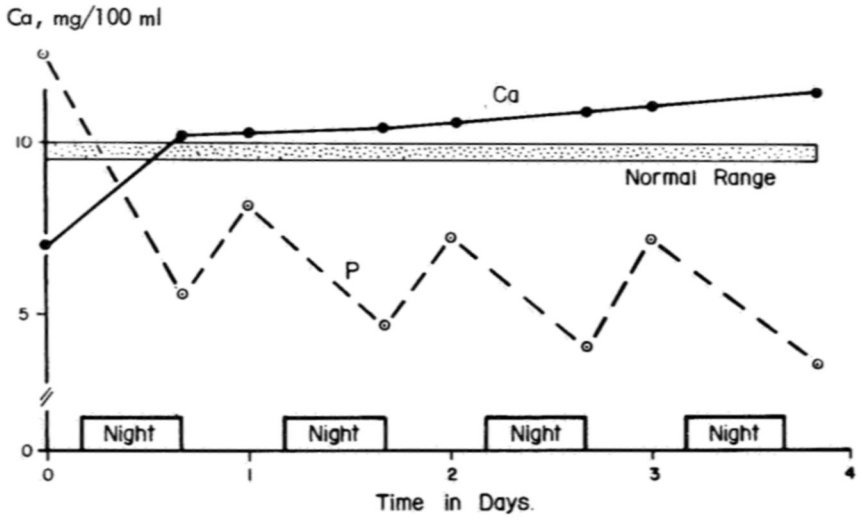


FIGURE 116. Changes in plasma calcium and phosphate when 5-week-old rats that had been thyroparathyroidectomized 24 hours earlier were fed a diet low in calcium (0.05 percent) and phosphorus (0.02 percent). Note the diurnal fluctuations in plasma phosphate. The points represent an average for seven rats.

observation that the response to both parathyroid hormone and thyrocalcitonin is greatly reduced in the rats which have been fed a phosphate-free diet. I should point out that these rats were not truly deficient because they had been fed the diet only for a few days at the most; thus,

the effects are more probably a result of the fall in blood phosphate concentration, which occurs almost immediately.

Feeding these month-old rats a diet with normal calcium content but free from phosphate resulted in hypercalcemia and hypophosphatemia within 24 hours. Even more interesting was the apparent effect on parathyroid function after 3 days on such a diet. The gland appeared to be nonfunctional; as shown in figure 117, the very slow fall in plasma calcium concentration that occurred during the day when the rats were fasting was essentially the same following parathyroidectomy as it was after sham operation. The similarity is even more striking (fig. 118) when hypocalcemia is produced in these animals by intraperitoneal injection of phosphate. The curves for the sham-operated and parathyroidectomized groups are the same, indicating essentially no homeostatic response to hypocalcemia.

TALMAGE: Have these rats been maintained on a low phosphate diet for only 3 days?

COPP: Yes, and I do not think there was time for any real deficiency changes in bone. In my opinion, the significant factor is the fall in plasma phosphate concentration.

We studied the effects of varying the levels of both calcium and phosphate in the diet on the response to parathyroid hormone and thyrocalcitonin, and the results are shown on table XXIV. The re-

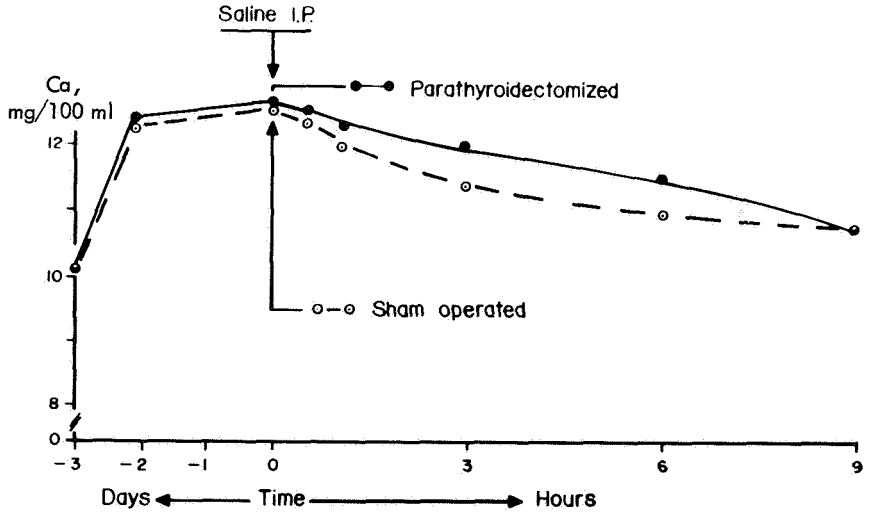


FIGURE 117. Changes in plasma calcium concentration in 5-week-old rats during the 3 days on the low phosphate diet and after parathyroidectomy or sham operation. The fall in calcium during the daytime fast is normal on this diet and is associated with an increase in plasma phosphate concentration. Note that removal of the parathyroids has essentially no effect.

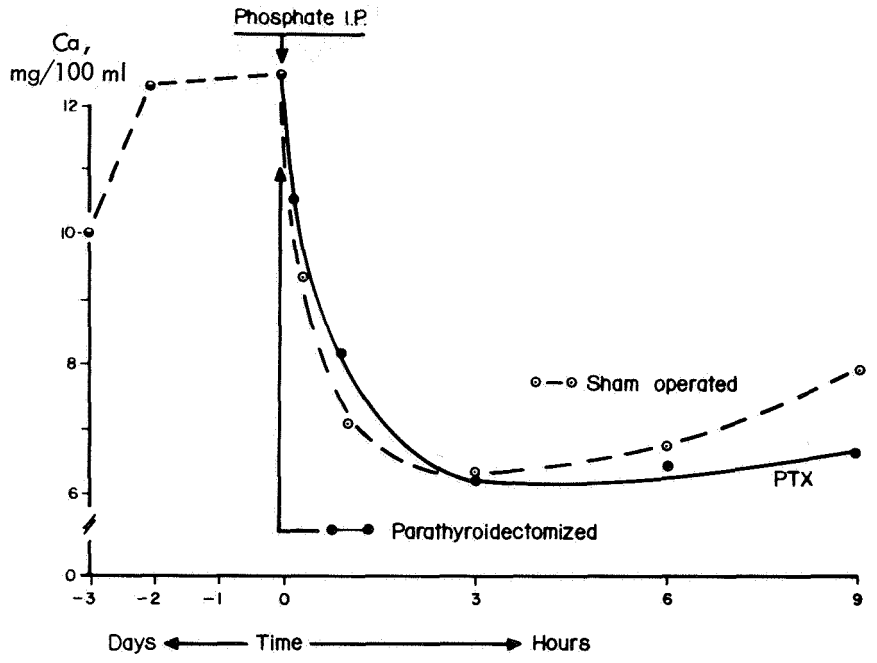


FIGURE 118. Changes in plasma calcium concentration in 5-week-old rats during the 3 days on the low phosphate diet and after intraperitoneal injection of inorganic phosphate and parathyroidectomy or sham operation. Note that there is essentially no homeostatic response for at least 6 hours, and the sham-operated animals behave in the same way as the parathyroidectomized group.

sponse to hormone was significantly reduced on the phosphate-free diet. This table gives a very reliable indication of response to the hormone and correlates very well with the logarithm of the dose administered in the case of either parathyroid hormone or thyrocalcitonin.

TABLE XXIV

EFFECT OF DIETARY CA AND P ON THE RESPONSE TO PTH AND TCT

	A	B	A	B	A	B
Dietary Ca, percent:						
0.80	3.1	2.10	6.5	9.5	17.5	10.7
0.40	7.4	2.30	18.0	9.6	9.2	10.6
0.01	1.6	2.8	11.1	16.7	8.5	15.2
Dietary P, percent	0.02		0.40		0.80	

NOTE.—Numbers represent area response between curve for plasma calcium concentration (mg/100 ml) against time (hours) after injection of parathyroid hormone (PTH) or thyrocalcitonin (TCT).

Col. A: area response to 1U of PTH/g; col. B: area response to 2 Hirsch U of TCT/g.

It will be seen that the responses to both hormones are significantly reduced in animals which are fed the phosphate-free diet. The best response to parathyroid hormone was obtained when there was adequate calcium and phosphate in the diet and the calcium-to-phosphate ratio was 1 to 1. The best thyrocalcitonin response was obtained with a calcium-free diet and adequate phosphate.

In conclusion I would like to refer again to figure 3 which showed the main factors involved in calcium homeostasis, including the enormous reservoir of calcium in the skeleton, and the two calciostats which control the plasma concentration in the normal animal: first, the parathyroid, releasing parathyroid hormone in response to hypocalcemia, and second, the thyroid, releasing thyrocalcitonin in response to hypercalcemia. The manner in which they may act is shown in figure 119. In this young dog, when the plasma calcium concentration was lowered by infusion of EDTA, the calcium rose rapidly after the infusion, presumably as a result of the action of endogenous parathyroid hormone released by the stimulus of hypocalcemia. However, the rapid increase stopped abruptly when the normal range was reached. This was presumably a result of the fast action of thyrocalcitonin,

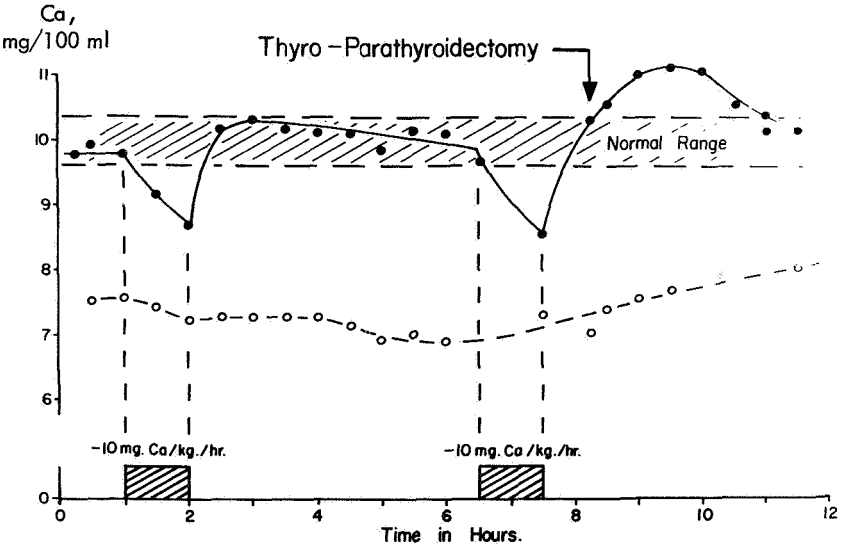


FIGURE 119. Evidence for the homeostatic role of the thyroid and parathyroid glands showing the recovery from EDTA-induced hypocalcemia with glands intact and in the same dog when thyroparathyroidectomy was performed (arrow). Note the overshoot in plasma calcium concentration, indicating loss of the very precise control of hypercalcemia when the glands are present. [Adapted from ref. 42; reprinted by permission of the publisher.]

because this sharp cutoff did not occur when the experiment was repeated and the thyroid and parathyroids were removed.

BAUER: May I register one protest? I think it is strange to show a scheme of this type and leave out vitamin D completely. There is considerable evidence that vitamin D has something to do with calcium metabolism.

COPP: I agree completely. Vitamin D is certainly a critical factor in calcium metabolism and I am sure that deficiency of this essential vitamin will interfere with normal calcium homeostasis. However, because of the limited time available, I hesitated to open this Pandora's box. Vitamin D could occupy a full session at some future time. We will put into the record that vitamin D has an important role in calcium metabolism.

BAUER: Two generations of children have been brought up on it.

URIST: We must stop at this point and go on with Dr. Owen's presentation.

OWEN: You have heard something about the various types of bone cells in a population. We have been looking at the pattern of RNA synthesis *in vivo* in the different cells of bone using autoradiographic techniques. The system we study is the actively growing periosteal surface of the midshaft of the femur of 1-week-old rabbits. The actively growing periosteal surface has characteristic loops representing forming primary osteons (fig. 120(*a*)). In figure 120(*b*) the loops close up to form canals in the process of bone growth, and each contains a blood vessel and some osteogenic cells. The osteoblasts, which form a single layer of cells on the bone surface, have the characteristic appearance of very active osteoblasts. Behind them is a thicker layer of cells which we have called preosteoblasts; these cells are the precursors of the osteoblasts. Behind these is a layer of fibroblasts about three cells thick which separates the osteogenic tissue from muscle. The osteocytes are embedded in the bone matrix. Within the haversian canals, cells which are on the bone surface have been called osteoblasts; cells within but not on the surface have been included in the category of preosteoblasts (fig. 120(*a*)).

In the previous work we have measured various parameters of this system and it was found that in these young actively growing animals the preosteoblasts and osteoblasts spend on the average of 3 days on the bone surface before becoming enclosed in bone, either as cells within the haversian canals or as osteocytes embedded in the bone matrix. The bone surface advances, in the process of bone growth, at the rate of about 70 microns per day, as illustrated in figure 120(*b*). It was thus possible to calculate the rate of production of matrix, and it was found that each osteoblast produces approximately its own volume of organic bone matrix per day during the 3-day period it spends

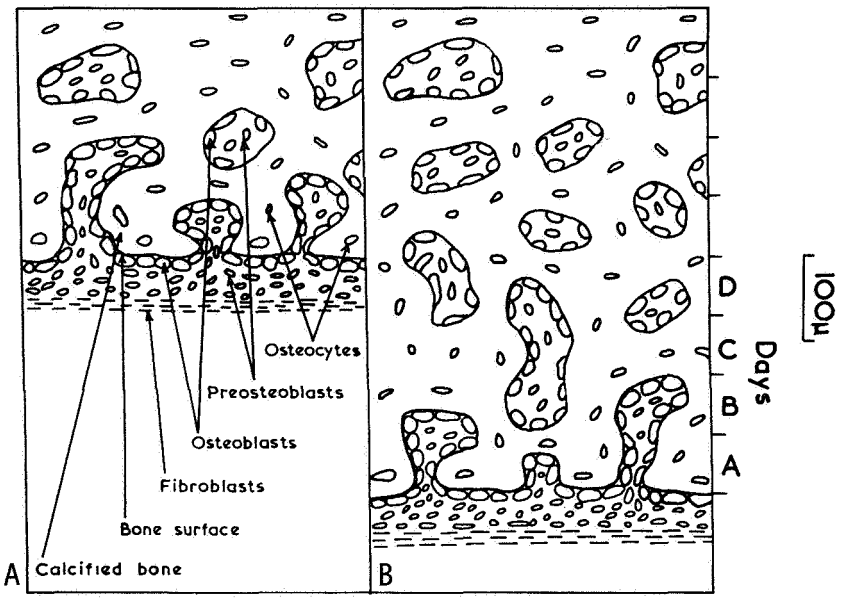


FIGURE 120. (*a*) Diagrammatic representation of part of the periosteal surface of a 1-week-old rabbit, the femur shaft. (*b*) The position after 4 days' growth.

on the periosteal bone surface. In addition, the endosteal bone surface in this region is lined with osteoclasts which are resorbing bone from this surface at approximately the same rate at which it is being laid down on the periosteal surface. A study of these osteoclasts was also made.

Here, then, is a system that includes different stages of cellular differentiation. First, the preosteoblasts engaged mainly in cell proliferation. Second, the osteoblasts on the bone surface, which are in a state of maximum functional activity, engaged in the synthesis of a few specific substances and in particular the protein, collagen. Third, the osteocytes and "haversian osteoblasts" which are a later and more quiescent state of the cell. Finally, the osteoclast which is responsible for bone resorption. It seemed of interest to investigate RNA synthesis and turnover in this system, particularly in relation to its pattern in the various cell types, representing, as they do, different stages of differentiation of the same cell.

Rabbits, 1 week old, were given a single intraperitoneal injection of ^{3}H-uridine as the RNA precursor and killed at different times after injection. The bones were fixed in 10 percent neutral formalin, decalcified, and embedded in paraffin. Sections, 5 microns thick, were cut

and autoradiographs prepared using the stripping-film technique. Counts were made of the number of grains per nucleus and per cytoplasm in the different cell types. The results up to 10 days after injection for the preosteoblasts and osteoblasts on the periosteal surface are shown in figure 121. It should be noted that the same pattern of labeling is followed both for preosteoblasts and osteoblasts; that is, there is an initial rapid appearance of label in the nucleus with a slower appearance of label in the cytoplasm. This is similar to the results obtained so far for other cell types. The curves for the nuclear labeling are almost identical for the preosteoblasts and osteoblasts (fig. 121); in the case of the cytoplasm the curves have a similar shape, but the level of labeling in the cytoplasm is lower in the preosteoblasts. The level of labeling in the cytoplasm does not reach its maximum until at least 18 hours after injection and it then falls off very slowly. The osteoblasts are most active during the 3 days which they spend on the bone surface, and are in fact producing, on the average, their own volume of protein per day during this period. An estimate of the rate

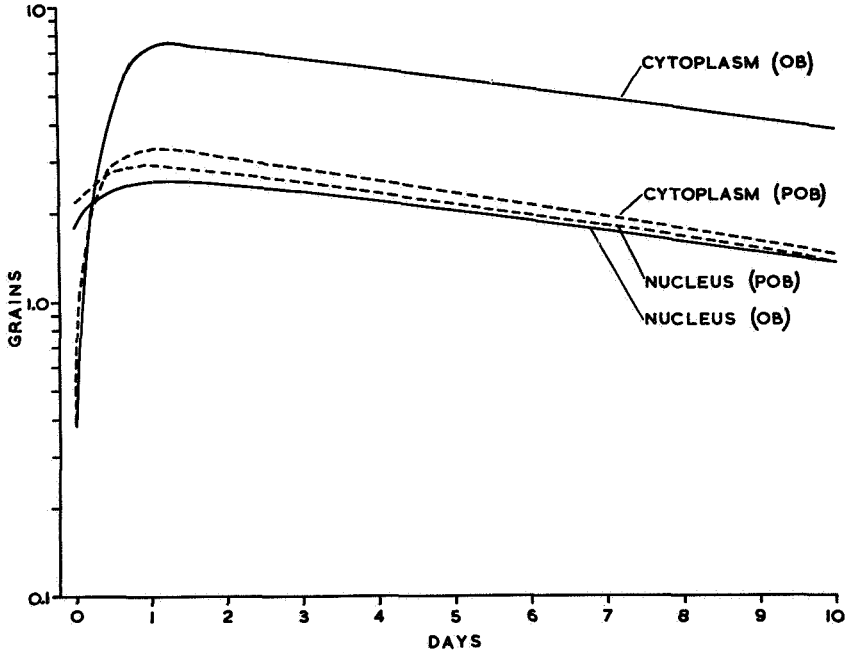


FIGURE 121. Grains per nucleus and per cytoplasm plotted against time after a single intraperitoneal injection of ^{3}H-uridine. The experimental points have not been included. The curves for nuclear labeling in the two cell types are almost identical. In the case of the cytoplasm, labeling is slower than in the nucleus and reaches a higher value in the osteoblast than in the preosteoblast.

of loss of RNA during this period can be determined from the falloff in the retention of labeled RNA between 1 and 4 days after injection. As can be seen from figure 121, the rate of loss of RNA during this time is about 8 percent per day.

Our results for the osteoclasts are not so complete. However, what results we have show that they follow a very similar pattern to those of the other two cell types. It is also of interest that we have found a difference in uptake in the osteoclasts in different regions of the bone, presumably depending on differences in osteoclastic activity.

PRITCHARD: Uptake of what?

OWEN: Tritiated uridine. Now I would like to mention the results for the osteocytes. We find two types of labeling of osteocytes. At short times after injection we see a low level of labeling in the osteocytes which are close to the growing bone surface. This can be explained in terms of a low rate of protein synthesis which continues in the most newly formed osteocytes after they have been embedded (ref. 112).

However, the second type of labeling, which will now be described, is of particular interest, since it illustrates an effect of environment on the functional apparatus of the cell. The fate of an osteoblast after its period of maximum activity on the bone surface is, in the process of bone growth, to become either an osteocyte embedded in bone matrix or an osteoblast on the surface of haversian canal (fig. 120(*b*)). Cells at any position *A* or *B*, for example, will be of the same age, and may be either osteocytes or "haversian osteoblasts." They will be radioactively labeled by virtue of the fact that they have taken up the radioactive precursor while they were osteoblasts on the surface at the time of injection. Figure 122 shows a comparison of the amount of RNA label remaining in these two types of cells of the same vintage, at three different positions—*A*, *B*, and *C*—in animals killed 3 or 4 days after injection. You will remember that position *A* was nearest the bone surface and these are the most recently formed osteocytes and "haversian osteoblasts." Positions *B* and *C* are deeper within the bone, respectively. The graph shows the ratio of the grains per osteocyte to the grains per osteoblast for the different positions. At position *A* the osteocyte has approximately the same grain count as the osteoblast, showing that it becomes embedded containing approximately the amount of RNA that it had as an osteoblast. At later times, i.e., at positions *B* and *C*, the osteocyte has lost its RNA, whereas the osteoblast retains its label. The implication of this result is that the osteocyte finding itself in a situation where it presumably is not going to need its protein synthesizing apparatus for some time is instructed under the effect of its new environment to abandon it. This result can be seen visually on a histologic section where the osteocytes near the growing

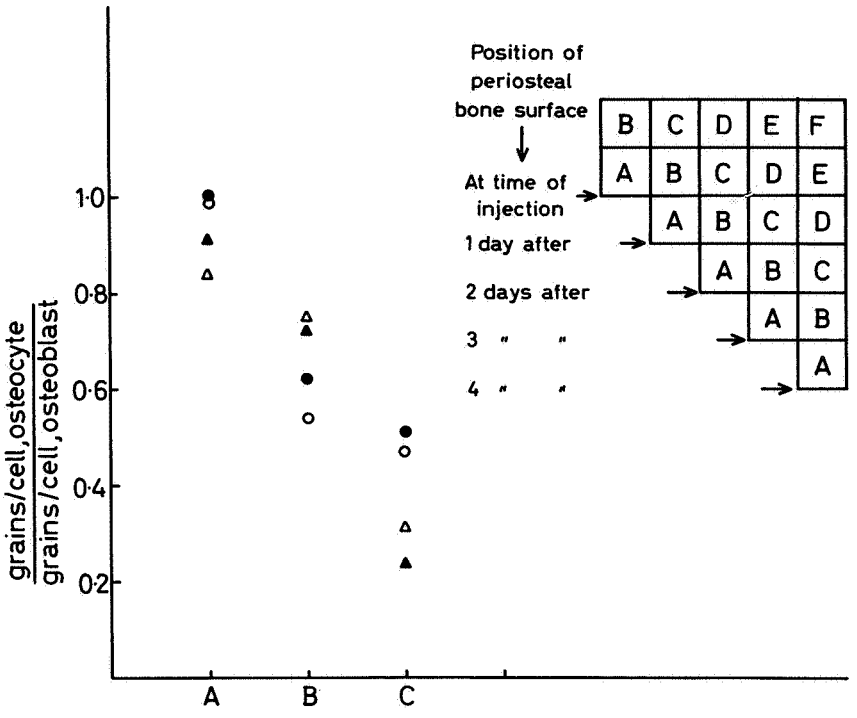
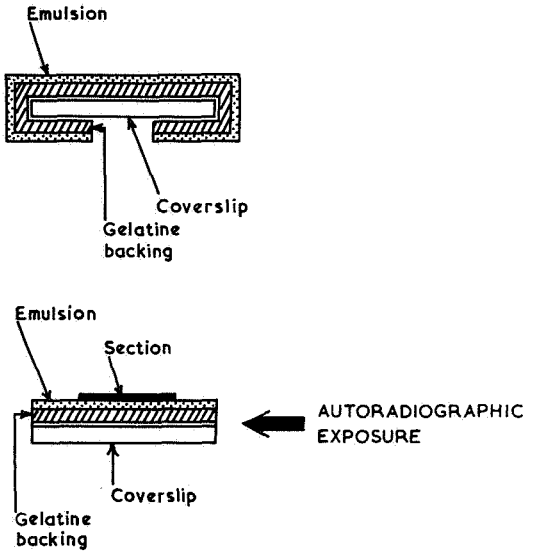


FIGURE 122. The ratio of grains per osteocyte to grains per osteoblast at different positions with reference to the bone surface. (See fig. 120.) The results are for four animals: three were killed at 3 days and one at 4 days after a single intraperitoneal injection of ^{3}H-uridine.

bone surface have cytoplasm full of RNA while there is a gradual loss of cytoplasm in osteocytes at increasing distance from the bone surface.

Finally, I would like to refer briefly to some other experiments that are relevant to the interpretation of the above results. The fact that one does not know how much of the label has been removed by the various histologic procedures is a limitation of the autoradiographic technique as used with paraffin sections. Recently we have made considerable progress in determining this, using the technique of frozen-section autoradiography which has not previously been applied to bone. Using this technique, it is possible to obtain autoradiographs of frozen sections in which the soluble components are retained. The technique is briefly as follows. Pieces of film were placed on a coverslip with the emulsion side up (fig. 123). These were cooled to $-20°$ C, the temperature of the cryostat. The bones were frozen in liquid nitrogen at $-180°$ C. Frozen sections were cut on the cryostat and picked up on the surface of the emulsion; this had to be done in the darkroom. This combination was then put away for autoradiographic exposure. After exposure the section was fixed and the film developed and the whole



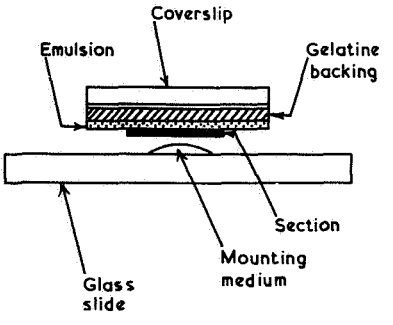


FIGURE 123. Diagram of the different steps in the production of an autoradiograph of a frozen section with the soluble components retained, using the method developed by Appleton (ref. 182).

combination inverted onto a slide for observation. In the process of fixation and development, of course, water-soluble components will be removed, but this does not matter since their image on the film has already been obtained (ref. 182).

It is possible to extract a frozen section to remove certain components and to compare the autoradiograph of the extracted section with that of a frozen section that has not been extracted. For this purpose we have extracted sections using the fixative, formol-acetic-alcohol solution, and compared these with autoradiographs of frozen sections where

all the components are retained. The radioactively labeled insoluble material remaining is considered to be representative of macromolecular RNA. The results show that at 1 hour after injection, only 14 percent of the label present is in RNA, the other 86 percent is washed out by the acid fixative. By 7 hours, about 50 percent is in RNA and by 24 hours it appears that a small fraction may still be extractable, although it is necessary to repeat the process to confirm this. It was also noted that more extractable material is washed out of the cytoplasm than out of the nucleus.

BÉLANGER: This is still uridine?

OWEN: Yes; we were using radioactive uridine in these experiments.

BÉLANGER: Do you interpret that as free uridine?

OWEN: It is probably labeled and has been incorporated into the RNA precursor pools, which contain small or intermediate-sized molecules.

BÉLANGER: It would not be just the label that is there?

OWEN: Yes; there could be some free uridine—I do not know. In time after injection, less of the label is in the extractable form and more is in the nonextractable form or macromolecular RNA. We are going to repeat and extend these experiments over the whole time scale.

I would like very briefly to give a quick résumé of the main points that I have made, which are relevant to the present discussion. The pattern of RNA synthesis and decay in osteoblasts and their precursors, the preosteoblasts, and also in the osteoclasts is qualitatively similar. The amount of RNA appearing in the cytoplasm, however, is greater in the osteoblast than in the preosteoblast. An interesting effect of environment on the retention of RNA was observed in the case of the osteocyte. Up to 24 hours after injection a significant fraction of the radioactive label is in an acid-extractable form.

RAISZ: I wanted to ask about the very slow loss of uridine from cells in your culture. I wonder if this represents reutilization of the label, since the decay rate for RNA for most tissues appears to be faster than this. The half times for uridine look to be 10 to 15 days in your material.

OWEN: Yes.

RAISZ: If this is true, this might explain that at 24 hours, the free material which you find actually represents a breakdown of reutilized uridine nucleotides or other soluble precursors.

OWEN: Yes; the half-life of RNA as measured from our curves is between 7 and 8 days. This is not so different from other measurements which have been made *in vivo;* for example, Loeb et al. (ref. 183) have found a value of about 5 days for the half-life of ribosomal RNA in rat liver. However, I agree with you that there is likely to be reutilization of RNA breakdown products and this is one of the problems in

interpreting RNA studies. I think further experiments using frozen-section autoradiography may give us some idea of how much of this occurs.

RAISZ: If this is so, then the decrease you see in the labeling of the osteocytes may be a function of stopping RNA synthesis and not of jettisoning the RNA of the cell. If reutilization has been maintaining the slow slope in the osteoblasts, the steeper slope in the osteocyte could simply represent decrease in synthesis rather than accelerated breakdown or actual secretion of RNA.

PRITCHARD: You have another bit of evidence. The osteocytes are smaller bodied, less basophilic, which is some indication of the total amount of RNA.

RAISZ: The question is whether it gets smaller by less synthesis or by getting rid of something.

PECK: I would like to compliment Dr. Owen on an elegant presentation. I think this is really fascinating work.

I have one question about the technique of washing. Binding of small amounts of tracer to macromolecules without actual incorporation is always a problem for the biochemist, and very often almost impossible to eliminate. I was wondering if you had tried, for example, adding stable uridine to the washing fluid to see whether this would make any difference.

OWEN: No; that would be worth doing, but I have not tried that. I have tried flushing out with stable uridine after injection of the labeled uridine. If I give an injection of stable uridine, hundredfold, 2 hours after injection of labeled uridine, it has no effect on my results.

URIST: I would like to turn the floor over to Dr. Currey for some concluding remarks to his earlier presentation.

CURREY: I am afraid I am going into the realm of the "wild-blue-yonder" theory—no elegant experiments, or anything like that. I want to talk briefly about the other aspects of local factors involved in remodeling, that is, how bone as a whole adapts itself by growth or remodeling, external remodeling, responding to unbalanced stresses that may fall on it; why femurs end up straight from one end to the other—that sort of question.

My thoughts on this were stimulated by Frost's book (ref. 184), which some of you may have read. He is rather overheavy on the mathematics of things, I think, but he certainly has some interesting ideas.

One has to consider under what circumstances bone needs to change its external form. I am going to use as an example an extremely simple system. I am only going to consider a long bone, without considering much about muscle pulls or the like—merely a long bone that should end up more or less straight.

If you have a long bone that is habitually (or more often than not) subjected to a simple bending stress, there is no particular alteration of its external form, except by increasing the total amount of bone tissue, that will make it any better at resisting that stress. In all cases, by increasing the total amount of bone tissue, you can, of course, reduce your liability to fracture, but one cannot go on increasing weight indefinitely, and presumably the safety factor cannot reach more than a certain level.

Similarly, if you have a bone which is loaded in pure compression, or in pure tension, there is nothing special that it can do to increase its resistance to fracture.

But when you get slightly more complicated systems, then there is something that the bone can do. For instance, suppose that the bone is loaded habitually in compression, and also is bent at the same time. If this bone is loaded habitually, more by being bent to the right rather than to the left (fig. 124(*a*)), then what the bone can do to reduce its liability to fracture is to become, when unloaded, bent to the left (fig. 124(*b*)). In other words, it should grow from being straight, into a bow. This perhaps may explain the slight forward bowing of the femur, for instance, because when it is loaded in compression it tends to bow forward even farther, and then the bending force of the adductors will tend to pull it back in, in the opposite direction, so the bending stress that would be produced by the adductors alone will be changed

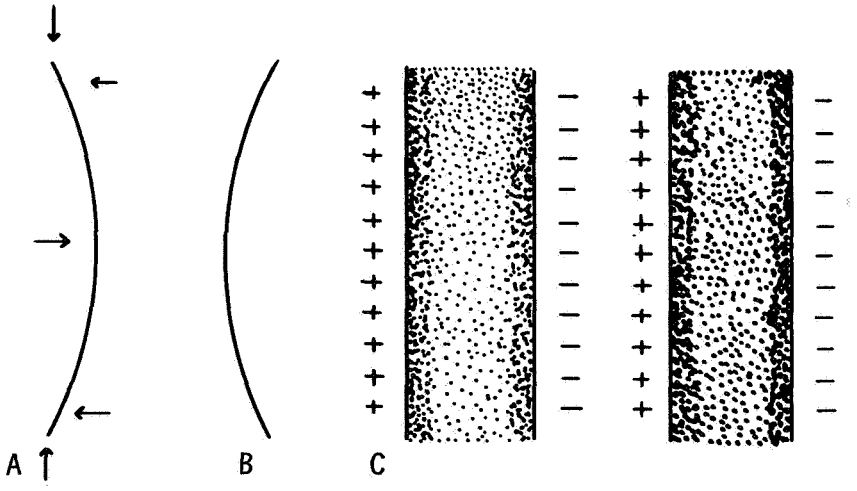


FIGURE 124. Long bone subjected to stress. (*a*) The bending produced by forces acting on an originally straight bone. (*b*) The shape of bone after adaptive growth that would counteract the bending forces. (*c*) Vertical section through the middle of the shaft, showing where bone must be added (+++) and where bone must be taken away (———).

into some slight increase in the compressive stress, and bones, of course, are good at resisting compression and not very good at resisting bending.

If you consider the shaft—the vertical section in figure 124(*c*)—to bring this adaptive bowing about, you have to resorb bone on the surfaces indicated by (−) and add bone on the surfaces indicated by (+).

Similarly, if you have a bone that is bent initially (fig. 125(*a*)) and is subjected to pure compression, the bowing itself is going to produce a bending moment; therefore, you want to straighten up (fig. 125(*b*)), and to do this, bone must be resorbed and added to.

Now, for such a relatively simple system, all that the bone—the accretion-resorption mechanism—needs to know at any particular point is that stress has occurred, and the change in the curvature of the surfaces.

FREMONT-SMITH: Stress has occurred, or stress is occurring?

CURREY: Both. One assumes it is going on the whole time. One has to integrate this over a certain time and, of course, living systems are very good at integration.

You also have to make a change in the curvature; and for the bone to appreciate that stress has occurred, it must be extremely simple. There must be hundreds of ways of doing this. On the other hand, to measure the change in curvature of a surface is very difficult indeed, particularly with the slight amount of strain that you will get in something like bone; but Frost (ref. 184) suggested that this is what is measured. The amount of change in curvature will be very small, but the change in strain through the thickness of the shaft can be measured. For example,

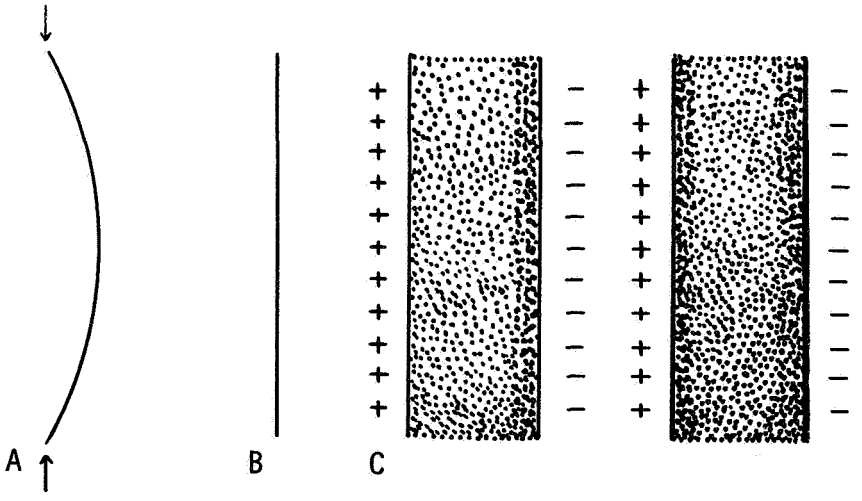


FIGURE 125. Same as figure 124, except that the bone is originally bowed to the right and is subjected only to longitudinal compression.

take the case shown in figure 124(*a*), where the bone is loaded axially and bent. Now let us look at the section through the cortex shown in figure 126(*a*). The left periosteal surface in this figure is going to be loaded in compression because of the axial stress, and additionally in compression because it is being bent out to the right, so we can represent the stress by three *C*'s on the left-top figure. Then the left endosteal surface is a bit nearer the neutral axis so that there will be a lower stress, represented on the right by two *C*'s.

The right endosteal surface is loaded in tension because of the bending, but there is also a longitudinal compression component. If you add these two—let us say for present purposes they add up to nothing—there is actually no stress at that endosteal surface, and the right periosteal surface is in tension because, although there is longitudinal compression, the bending more than counteracts it.

If you draw a horizontal line above which there is compression and below which there is tension, then you have high compression on the left, and tension on the right (see fig. 126(*b*)). In other words, there is a change in the compression or tension as you go in deep from a surface. Therefore, if you can measure the change in the state of

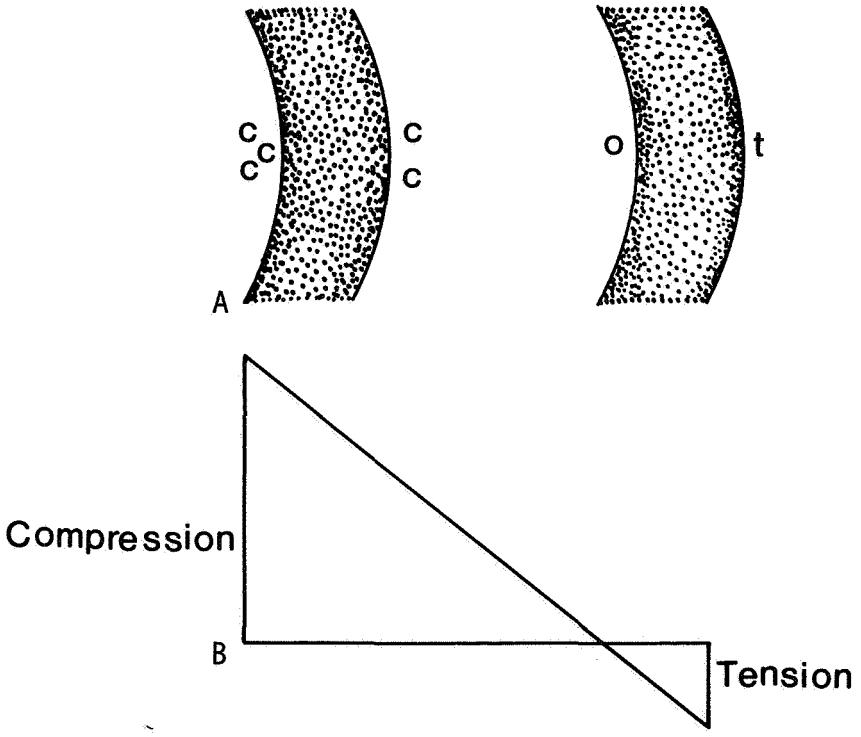


FIGURE 126. The state of stress in a longitudinal section of a bent bone.

stress as you go into the bone from a surface, you can, in effect, measure the change of curvature that has occurred at that surface. And with the system that I have talked about so far, you could devise a simple rule, and that is: Take away bone from a surface if the strain gets more compressive as you go in, and vice versa.

This is Frost's system (ref. 184). The modification I have introduced is not measuring surface curvature which is probably impossible, but measuring changes in stress as you go in deep. This works for both endosteal and periosteal surfaces. Bassett (ref. 185) produced a simple scheme which enabled his electric phenomena to explain changes in adaptive growth, and if you followed his system and worked out what happened, you would find that you would get one side of the bone disappearing entirely and the other side increasing in width; this would not be a satisfactory arrangement. You must have a system that can tell the endosteal surface to behave in a different way from the periosteal surface.

How do we measure this change in stress? I do not know, but Bassett and Becker (ref. 186), and others, have shown that electric potential generated by the stress would seem a very simple way of measuring stress. The canaliculi in bone, of course, making connection with each other in depth seem to be absolutely tailormade for measuring changes in stress, or maybe ionic fluxes over some little distance of the order perhaps of even a millimeter, which would be quite sufficient.

The system of Frost (ref. 184),) with the modification that I have introduced, would work perfectly well with the rather simplified system I have used if bone were never loaded in tension as a whole. The bone was loaded in compression axially, but because of bending, the right side was loaded in tension and the left side might have been very much in compression, but if you add up all of the stresses of a particular section and divide by the number of places you measured, you find it comes to compression.

If you ever have a net tensile stress on a long bone, then this system of Frost's will produce the wrong answer. If, for example, you have a bent bone loaded in tension, you get the wrong answer; you get an opposing feedback system, with more and more bend until the bone breaks. So this will not work if bones are loaded in tension.

PRITCHARD: There are one or two other things. You have left out the periosteum, which is under tension and which could itself be a receptor.

CURREY: Yes, true.

PRITCHARD: And you could get a feedback from the periosteum.

CURREY: Yes.

PRITCHARD: Another factor again is reflex contraction which will tend to reduce tension and replace it by compression. Bone is not just a piece of wood.

CURREY: I am not thinking for a moment that it is. Could I take those points separately? We will take the second one first and say that I quite agree that muscles, of course, will reduce unbalanced stresses a lot, but there will probably be residual unbalanced stresses, particularly if the bone is the wrong shape.

With reference to the periosteum, I think the periosteum is very important. However, what the periosteum cannot do on its own is measure a change of stress with depth. I will bring in the periosteum directly.

If we have to consider loading in tension, what you find is that with this simple rule I have suggested about taking bone away, you get more compression; if this works, if the bone is loaded in net compression, and if the bone is loaded with net tension, then you have simply to reverse the rule.

When I first came across this, I thought, "Now we can just work out a way in which you can reverse the rule." All you have to do for the section as a whole is to know whether it is loaded under net tension or net compression. This is extremely difficult, because what it means is that any particular part of the bone has to get information in some way about the stress acting on the bone as a whole. One cannot do it by any simple method of measuring the flux at a particular point of measurement. You have to get information which comes from other parts of the bone, and possibly this is where the periosteum will come into play.

The minimum information required, in more or less symmetric circular bone, would be, at any particular point on the bone, to have information about the stress there and the stress diametrically opposite. If you just add the stress at these two points and take the mean, as it were, this will tell you whether there is net compression or tension.

But it seems to me somewhat unlikely that you have rather neat nerves going around to diametrically opposite parts of the bone, and what I would like to inquire of you who know something about histology of bone is whether anything in detail is known about the arrangement of the nerve fibers in the periosteum, and to some extent in the endosteum.

All I have shown here is the minimum amount of information that is required for a system to change the shape of a bone in an adaptive fashion. It sounds quite small. We just want to know the change of stress at depth, and the net stress on the whole tension. In fact, I think the practical difficulties are going to be rather large.

ROBINSON: The point is brought up that if you are going to try to convert mechanical energy into cell activity, you need some sort of

receptor system, and there is a system of nerves in the body which is, I think, often overlooked. The system of C-fibers is a very positive system (Sir Thomas Lewis (ref. 187), discussed this in his book on pain), because pain does arise from the deep somatic structures, and we are constantly faced with the presence of this system. We do not know exactly how it works, but nevertheless it is there.

Dr. Milgram, a student who was working with us on the cortex of the femur of dogs, trying to cut haversian canals, and Dr. Cooper, similarly employed, suddenly ran into the presence of nerves in haversian canals (refs. 25 and 188). This has been described before with certain histologic techniques, but here we have definite morphologic evidence that such nerves are present in the haversian canals of dogs.

The adult animal has a paucity of osteoblasts in the haversian region. However, in the area such as the one shown in figure 127, between the osteoblast and the endothelial cell, one sees a Schwann cell. This is more than occasional. I should say they are found in more than half of the canals in the adult animal in the middle of the femur; that is, halfway between the periosteum and the endosteum of the cortex.

Figure 128 is an excellent electron micrograph by Dr. Cooper and is of higher magnification than figure 127. It shows part of two endothelial cells of the capillary in this haversian canal; the basement membrane is clearly seen. The adjacent Schwann cell has its own basement membrane. The small C-fibers are invaginated into the Schwann cell's plasma membrane. One can see bundles of collagen fibrils in this region around the Schwann cell. Although the Schwann cell lies between an endothelial cell and an osteoblast, we have never observed the nerve fibers going to endothelial cells or osteoblasts in haversian canals of the femur of the adult dog. We have never observed these nerves at all in the young growing puppy.

FREMONT-SMITH: Have you done any nerve degeneration studies?

ROBINSON: Yes, Dr. Milgram is now working on this.

FREMONT-SMITH: This ought to give a very sharp clue, ought it not?

ROBINSON: We hope that it will. Of course, we feel that it has implications in, for instance, Paget's disease, where it appears that the cells are not behaving in any orderly, controlled fashion in response to stresses and strains.

BÉLANGER: May I ask Dr. Robinson to elaborate on how the nerves would influence Paget's disease? Is that a vascular change that you expect, or what? Are these autonomic nerves?

ROBINSON: This is pure speculation.

BÉLANGER: Are they autonomic nerves, do you think?

ROBINSON: This is the type of nerve fiber which is associated with deep somatic innervation. The same type of fiber is seen in the various ligaments; for instance, the anterior and posterior longitudinal ligament

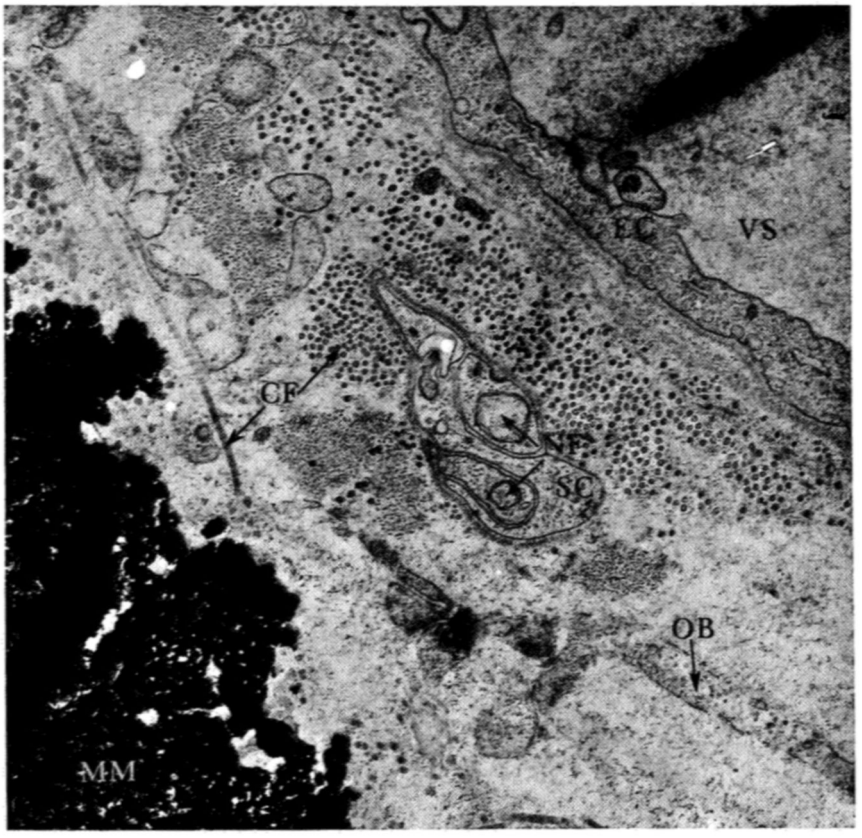


FIGURE 127. Electron micrograph of the space between the endothelial cell (*EC*) wall of the haversian vessel and the mineralized bone matrix (*MM*) at the periphery of a completed haversian canal from the femoral cortex of a dog. In the upper right corner is the vascular space (*VS*). Pinocytic vacuoles are seen in the endothelial cell wall. A junction between the endothelial cells is seen at the top of the picture. The separate basement membrane is observed along the avascular side of the endothelial cells and around the Schwann cell (*SC*). The nerve fibers (*NF*) are invaginated into the Schwann cell. Collagen fibrils (*CF*) are scattered throughout the extracellular, extravascular fluid space between the endothelial cells and the mineralized bone matrix. The osteoblast (*OB*) cell cover of the bone matrix seems fenestrated and incomplete.

In this preparation, the collagen fibrils stained more densely than the cement substance, unlike those in figure 14. Moreover, critical inspection of the mineral deposits on the periphery of the mineralized bone matrix suggests that the primary mineralization here may be in the collagen fibrils rather than between them, as in figure 14. Eventually, both the fibrils and space between are obliterated by mineral crystal density (left lower corner). Approximately 23 600 ×.

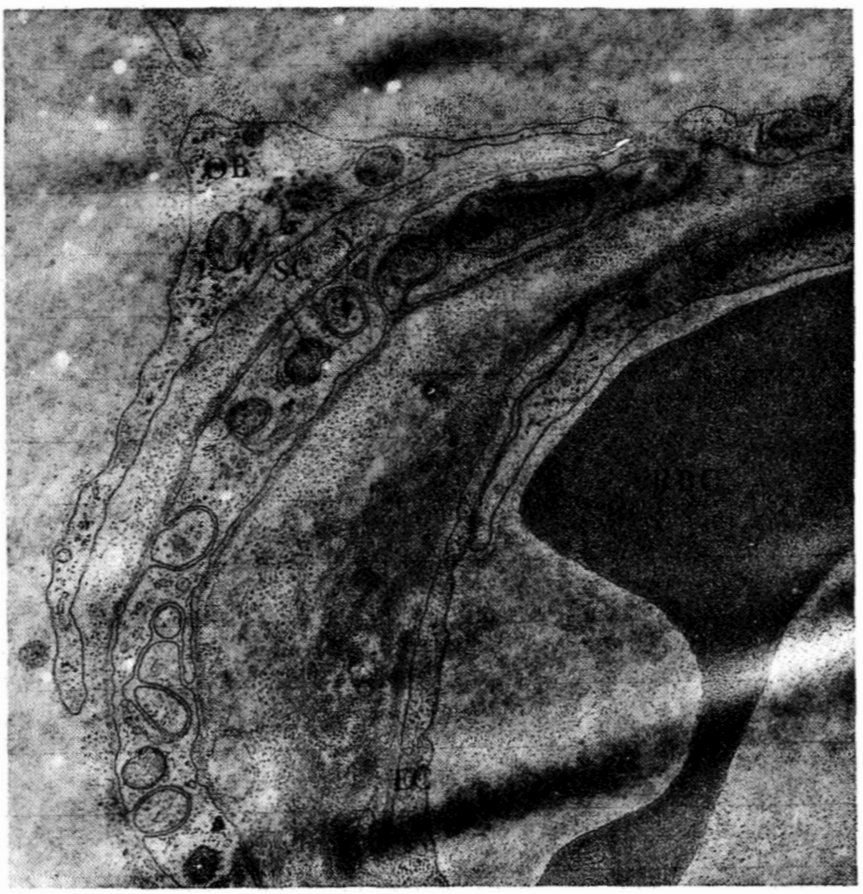


FIGURE 128. Electron micrograph of a section from the femoral cortex of a dog showing, in detail, parts of two endothelial cells (*EC*) and a Schwann cell (*SC*) with eight nerve fibers (*N*) invaginated simply into its plasma membrane. In the specialized regions, i.e., between the plasma membrane of two endothelial cells, there are usually two of these desmosome-like regions between the junction of two cells in the capillaries of the haversian canals in dogs. Osteoblast (*OB*) atrophied, or resting, in this instance; red blood cell (*RBC*) in lumen of haversian capillary; intermediate cell (*IC*). Approximately 27 200×.

of the spine, the annulus of the disk. This type also accompanies the small vessels which permeate our whole system. Perhaps I should point out the difference between this nerve and the myelinated nerve. Would that be helpful?

BÉLANGER: Yes.

ROBINSON: It has been clearly demonstrated by Robertson (ref. 189) that a myelinated or A-nerve fiber varies not only in size, having gen-

erally a much greater diameter than C-fibers, but also in the way it is invaginated into the Schwann cell's plasma membrane; it is wrapped in a spiral invagination of the plasma membrane of the Schwann cell. Since the plasma membrane is a lipoprotein, its multiple layering about the A-fibers forms the myelin cover of those nerves. Thus, by size and by the type of invagination into the Schwann cell one can morphologically distinguish the myelinated A-fibers from the smaller unmyelinated fibers of which the C-system is composed.

URIST: Thank you. We will have another presentation on cell physiology, and as soon as we get some cell physiology into this information-and-control concept, we will return to the subject of cellular differentiation. I am going to ask Dr. Howell to proceed.

HOWELL: For the past 6 years we have been studying processes involved in calcification of epiphyseal cartilage in normal and rachitic calves as well as in normal and rachitic rats.

The part of our recent work which I thought might be of interest has to do with a new system we have developed for studying the epiphyseal plate cartilage with respect to a fluid phase which we have been able to aspirate from the cartilage.

URIST: By micropuncture?

HOWELL: Yes. Earlier in the conference, regulation of mineral-phase transformation was discussed by Dr. Nichols and Dr. Bélanger, and views were advanced that certain key events might mediate osteoblastic regulation of mineral deposition in bone, including, among other possibilities, active transport of mineral ions into the mineralizing site, removal of water from the site, increase of pH, or formation of nucleation centers. To obtain direct evidence for such regulator factors and to evaluate their relative importance—if multiple factors are involved—is now virtually impossible in bone. However, for a similar biologic system, we have for the first time been able to sample and study a fluid phase; i.e., in hypertrophic cell cartilage of rachitic rats before and during calcification *in vivo*. I would like to present briefly some data on this preparation because they offer potentials for directly testing mechanisms of mineral-phase separation as influenced, for example, by parathyroid hormone, thyrocalcitonin, or vitamin D.

Our experimental preparation is illustrated in figure 129. A normal or rachitic rat under Nembutal anesthesia is placed on a plastic operating table similar to that used for renal micropuncture studies. The limb is fixed in 45° flexion and bathed with oil at 37° C. A finely adjustable vacuum pump is used to control an oil column which is communicated to the surface of the cartilage through the micropipet. As you know, in renal micropuncture, micropipets with tips of 5 to 10 microns are placed on a manipulator and guided under direct visual-

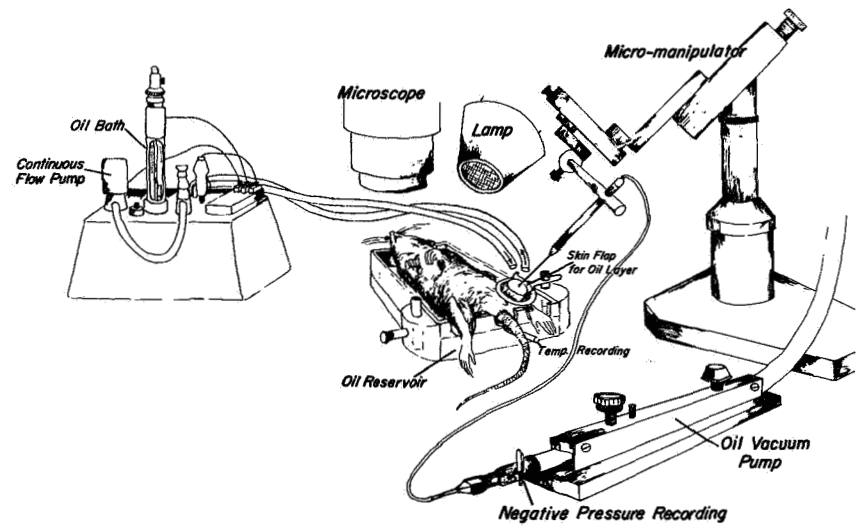


FIGURE 129. Assembly for use of cartilage micropuncture technique. [From ref. 190; reprinted by permission of the publisher.]

ization into renal tubules; fluid is aspirated and samples studied with respect to various parameters of renal tubular transport.

Here we employed the same approach to obtain fluid from the cartilage plate. We aspirate in exactly the same manner. The operative stress of this procedure is less than that resulting from renal micropuncture. Only a nick is made medial to the extensor tendon, and our open tissue exposure is small (ref. 190).

FREMONT-SMITH: Where do you get the fluid? Do you get the fluid from that device?

HOWELL: We have evidence that the micropipet tips are placed between the rows of hypertrophic cells of the cartilage plate. Fluid that is obtained from such a site is clear and shows no fibers or cells when observed under a phase microscope. The volume is from 20 to 40 millimicroliters, collected during 10 or 15 minutes at a negative pressure sufficient to overcome tip resistance—300 millimeters Hg.

This fluid is obtained easily, but is of such small volume that we had to devise ultramicro methods for analysis of calcium, phosphate, protein, and nucleotides. For this purpose, we utilized a Zeiss PMQ II spectrophotometer with a Zeiss No. 507425 special sample changer; we constructed a Bakelite positioner and an ultramicro cuvette (fig. 130) of 8.4-microliter total capacity (ref. 191). With this cuvette, direct quantitation of these parameters down to level 10^{-10} grams has been achieved with a satisfactory range of recoveries (refs. 190 and 191).

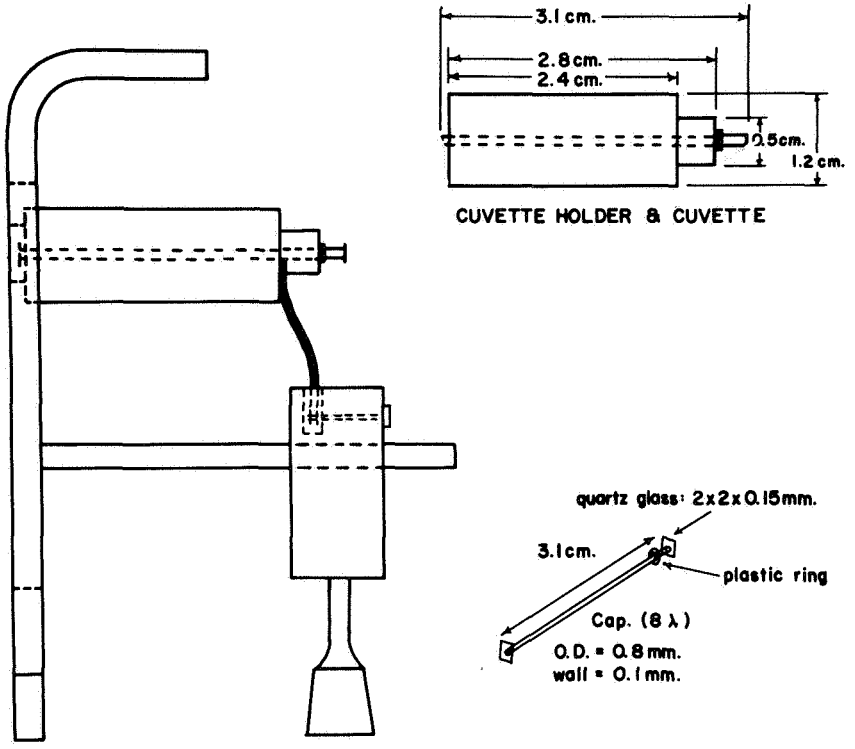


FIGURE 130. Ultramicro cuvette and positioner employed for spectrophotometric analysis of calcium and other parameters of microscopic cartilage fluid samples. [From Howell et al., ref. 191; reprinted by permission of the publisher.]

Now let us consider the question of the site of fluid collection posed by Dr. Fremont-Smith. After collection of samples, we replace a micropipet in the cartilage with the manipulator in the same position and inject a ferric chloride marker contained in Carbowax. Histologic sections are made from the collection sites and these are subjected to Perl's reaction for iron (fig. 131). The exact site of obtaining fluid is estimated from relevant histologic sections. Thus, we have obtained the hypertrophic cartilage cell fluid samples, designated henceforth as Fl_{SA}, less than 0.5 millimeter deep to the cut made in the surface of the cartilage plate and less than 0.2 millimeter distal to the junction between cartilage plate and bone epiphysis (fig. 132). The site of fluid collection from the resting cell cartilage, Fl_{SB}, was about 0.2 millimeter or less from the surface cut.

Next, we determine that the fluid Fl_{SA} was not derived from perichondrial surface fluid leakage. To do this, we placed a 1-percent solution of Iodocyamine Green in 0.9 percent saline on the nicked perichondrial surface of the cartilage and made routine aspirations of

FIGURE 131. Photomicrograph of rat upper tibial epiphyseal plate. Approximate site of collection of microscopic fluid samples assessed by injection through micropipet of a marker indicated at point of arrow. Phloxine stain. 120×.

Fl_{SA} from the hypertrophic cell region after varying periods up to one-half hour (fig. 133). The ultramicro cuvette readings for these aspirated samples indicated less than 4 percent contamination with the dye-containing surface fluid (ref. 190).

We also made consecutive attempts to obtain fluid from tissue sites adjacent to hypertrophic cell cartilage represented in figure 134. The percentages of attempted micropuncture aspirations that were successful in obtaining a clear fluid were: 90 for the hypertrophic cell region; 75 for the resting-cell cartilage next to the perichondrium; and 95 for the perichondrial tissue and muscle insertion sites. No fluid could be aspirated from resting-cell or articular cartilage, and only

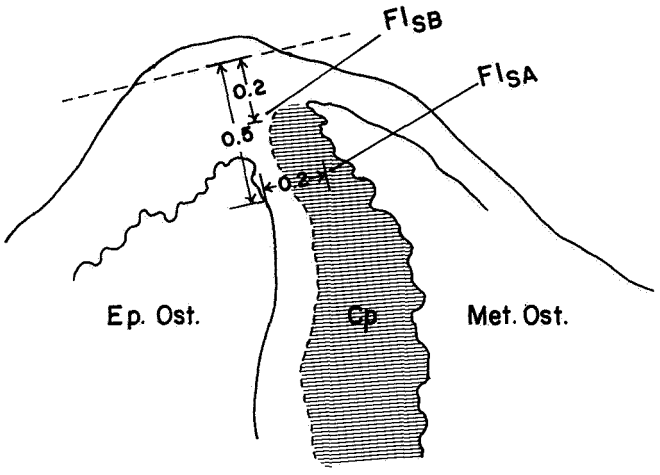


FIGURE 132. Collection site determined from a study of serial histologic sections of tissue, such as illustrated by figure 131, during the insertion of micropipet. Fl_{SA} indicates site of hypertrophic cell cartilage fluid collection; Fl_{SB} designates site of fluid from resting cell cartilage near surface; cartilage plate, Cp; epiphysis, Ep. Ost.; metaphysis, Met. Ost.

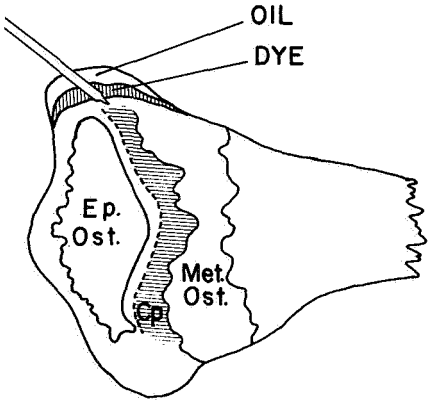


FIGURE 133. Diagram indicating position of micropipet during collection. In experiments with Iodocyamine Green, contamination of Fl_{SA} was less than 4 percent. Cartilage plate, Cp; metaphysis, Met. Ost.; epiphysis, Ep. Ost.

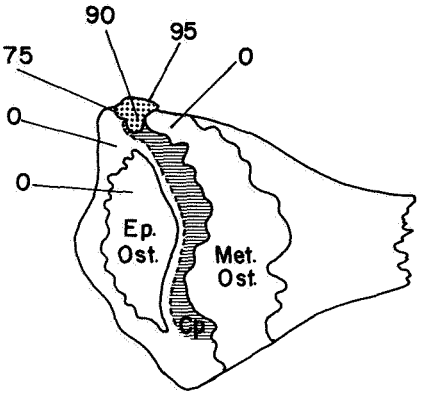


FIGURE 134. Percentage of attempted punctures, in serial trials (15–26 rats per tissue site), that yielded a satisfactory fluid sample. Cartilage plate, Cp; epiphysis, Ep. Ost.; metaphysis, Met. Ost.

blood was obtained from bony tissues. Brief mention should be made of other experiments on the fluid origin. Successful fluid collections were made from hypertrophic cell cartilage dissected free from the metaphysis and incubated in a concave slide under oil. The experiments indicated that most of the fluid was drawn from the hypertrophic cell cartilage per se and not aspirated through channels in the cartilage from a distant site (ref. 190).

Subsequent efforts were directed to ascertaining whether Fl_{SA} represented intracellular fluid, a result of cell damage, rather than a phase bathing the extracellular matrix. Chloride was quantitated in Fl_{SA} for 10- to 20-millimicroliter volumes (table XXV). Such fluid was found to be essentially extracellular due to its chloride content averaging about 100 mEq/l, corrected for protein.

FREMONT-SMITH: What is the total protein content? This could tell us whether or not it is really like plasma.

HOWELL: Total protein averaged a little over 7 g/100 ml in serum, 3 g/100 ml in Fl_{SA}, and 1 g/100 ml in Fl_{SB}.

FREMONT-SMITH: Did you ever get any fluid from normal rats?

HOWELL: Despite technical difficulties due to the narrowness of the hypertrophic cell cartilage of normal endochondral plates, we have recently succeeded in making such aspirations. In preliminary data, the protein content is about 2.5 g/100 ml. We have substantial data on the normal epiphyseal plate.

FREMONT-SMITH: The fluid in the normal is more like an edema

TABLE XXV

ANALYSIS OF MICROSCOPIC FLUID SAMPLES (Fl_{SA}) ASPIRATED BY MICROPUNCTURE FROM PLATE CARTILAGE OF UNTREATED RACHITIC RATS

	Units	Number of animals	Fl_{SA}	Serum
Chloride	mEq/l	8	[a]100±2	107±3
Protein	g/100 ml	15	3.1±0.2	7.0±0.5
Nucleotides	mg/100 ml	4	80±15	18.0±9
Hemoglobin	mg/100 ml	25	< 50	< 50
Calcium	mmoles/l	12	1.95±0.10	2.40±0.12
Pi_{TCA}	mmoles/l	15	2.20±0.2	0.65±0.1

[a] Standard deviation; applies to tables XXV to XXVII.

fluid with very low protein, and this fluid that you are sampling is about half-diluted plasma, so far as protein is concerned.

HOWELL: Possibly. However, the fluid appears to be much more viscous than plasma manipulated in the same volume under oil and one suspects some differences in composition.

PRITCHARD: Is the hypertrophic cartilage in rickets normal?

HOWELL: No differences from normal in the utilization of pyruvate, glucose, and lactate by cartilage slices *in vitro* from similar phosphate-depleted rachitic animals has been shown in the studies of Kunin and Krane (refs. 192 and 193). Such metabolic alterations are restored toward normal in their studies by phosphate repletion with or without vitamin D. However, during such phosphate repletion *in vivo* or during incubations *in vitro*, mineral-phase separation appears discretely within the septa of hypertrophic cell cartilage, as in the normal plate.

With respect to characterizing Fl_{SA}, as listed in table XXV, the level of nucleotides is higher than that of serum, as estimated from the 260/280-millimicron ratio correlated with total protein (ref. 190). There was no detectable hemoglobin, and total calcium was approximately two-thirds of the plasma level. A striking positive finding was the concentration of the inorganic phosphate Pi_{TCA} in Fl_{SA}, a level twice that in the serum. Our subscript for this parameter is important, since it indicates that histidine-linked or other acid-labile phosphates would register in total values for inorganic phosphate (ref. 194).

URIST: Does the high level of phosphate occur in the animal with low-phosphorus, vitamin-D-deficiency rickets?

HOWELL: Yes; the plasma phosphate concentrations indicated a severe degree of rickets. Two preparations of interest have come out of studying this fluid. We have incubated slices of cartilage prepara-

tions in calcium phosphate solutions (Yendt's solution) for 18 hours in a manner dictated by the studies of Sobel et al. (ref. 195), where they attempt to define early preformed mineral in the cartilage slices. I will not call them crystal nuclei, but some early mineral form is identified, they believe, if one incubates these cartilage slices in iodoacetate and magnesium for 18 hours. This was done in our studies, and correlations were made between the level of phosphate in the fluid and the histologic appearance of these slices. After studying about 14 different experimental preparations of the cartilage of rats treated in different ways by different dosages of vitamin D and varying phosphate intake, we found that with the administration of sodium phosphate, first done by Dr. Robinson here, I believe——

URIST: It actually was first done by Dr. McLean, on rachitic rats. He reported this experiment in which he gave solutions of phosphate to phosphorus-deficient rats, and demonstrated that he could initiate calcification in the epiphyseal cartilage without any vitamin D.

MCLEAN: It was presented before the Biochemical Society at a federation meeting (ref. 196).

URIST: My first experiment in his laboratory was to inject phosphate buffer in rats with healing fractures. We made observations on the callus of healing fractures in these phosphorus-deficient rats (ref. 197).

HOWELL: Excellent. In any event, after 2 hours of giving sodium phosphate preparation to a rachitic rat, the cartilage under electron microscopy did not show anything except staggered early dense areas suggesting mineral. After incubation for 18 hours——

URIST: Incubated in what?

HOWELL: Two percent of Yendt's solution (ref. 198), at a Ca times P product of 45 mg^2 percent, in the presence of magnesium and iodoacetate, showing, we believe, some alteration had occurred in the matrix as a result of having given the sodium phosphate. The interesting feature is that we have been unable to show any rise of calcium phosphate in this cartilage fluid.

Subsequently, rachitic rats were given an oral dose of 1500 units of vitamin D and Fl_{SA} samples at 0, 2, and 24 hours. During early stages of healing, inorganic phosphate increased in plasma and Fl_{SA} with no detectable alterations of the high Fl_{SA}/serum ratio (fig. 135). A similar study of the calcium concentration of Fl_{SA} in littermates treated in the same manner showed a slight and inconsistent increase (fig. 136). In this experiment all animals, including controls, had mild rickets due to a boost of serum phosphate from dietary privation 2 to 3 days previously and mineral appeared rapidly as evidenced by positive von Kóssa histologic sections of cartilage plates.

Having the total calcium and inorganic phosphate concentrations of cartilage fluids for this and a variety of other experimental rachito-

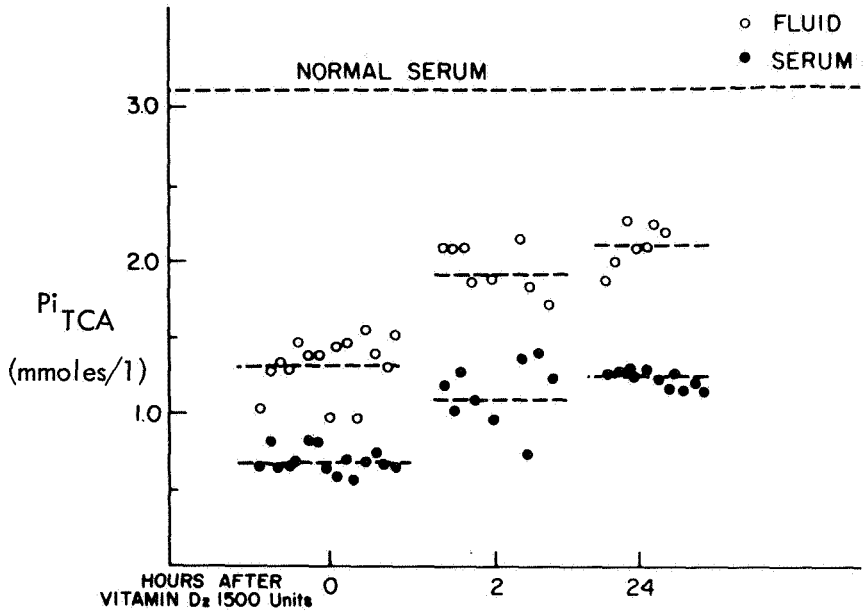


FIGURE 135. Inorganic (and acid-labile) phosphate, Pi_{TCA}, of hypertrophic cell cartilage fluid obtained by micropuncture (Fl_{SA}) before and after treatment with vitamin D. Each open circle indicates a Fl_{SA} value for one rat, and each corresponding closed circle, serum value for the same animal. Dashed lines represent mean serum values for 10 untreated normal control rats of the same age.

genic regimens, it was relevant to assess the nonprotein-bound calcium and phosphate. As of now, we have data on animals with severe rickets (table XXVI). For this measurement, we scaled down the method of Loken et al. (ref. 199), wherein serum-protein-bound calcium is assessed following ultracentrifugation. A special holder (fig. 137) was fitted to the Model L Spinco ultracentrifuge. Micropipets containing 20 millimicroliters of original cartilage fluid under CO_2-equilibrated oil were prepared with a bubble containing nitrogen and 5 percent CO_2 in the tip, sealed, mounted in the holder, and centrifuged at 105 000 g for 12 hours at 12° C. Following centrifugation, the sediment, together with the tip, was cut off under the dissecting microscope and supernatants analyzed for calcium and phosphate. Alternate samples were analyzed for pH, using an ultramicro pH glass electrode assembly with measurements under CO_2-equilibrated oil (ref. 190).

Rat serum centrifuged in this manner with a 20-millimicroliter starting sample provided a level of about 60 percent nonprotein-bound calcium, whereas the cartilage fluid (Fl_{SA}) level was about 65 percent, at the pH levels listed in table XXVII. Protein concentrations in the

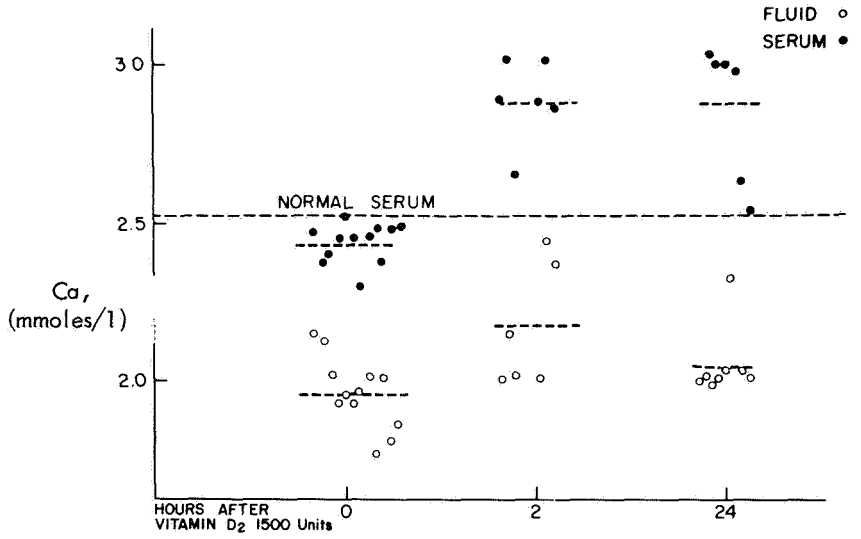


FIGURE 136. Total calcium concentration for serum and Fl_{SA} of littermates of rats in same experiments as figure 135. Each open circle indicates a Fl_{SA} value for one rat, and each corresponding closed circle, serum value for the same animal. Dashed lines represent mean serum values for 10 untreated normal control rats of the same age.

TABLE XXVI

TOTAL CALCIUM, NONPROTEIN-BOUND CALCIUM, PROTEIN, AND pH OF RACHITIC RAT SERUM AND CARTILAGE FLUID (Fl_{SA})[a]

	Serum (20 mμl)	Fl_{SA} (20 mμl)
Total calcium (mmoles/l)	2.40 ± 0.13	1.95 ± 0.10
Supernatant calcium (mmoles/l)	1.41 ± 0.05	1.26 ± 0.05
Estimate percent of nonprotein-bound calcium	59 ± 3.0	65 ± 4.5
Protein (g/100 ml)	7.0 ± 0.3	3.1 ± 0.2
Final pH	7.5 ± 0.1	7.7 ± 0.3

[a] Total values are for 12 animals, and supernatant values for 9. The pH figures after ultracentrifugation bear no certain relationship to fresh fluid values.

fluid decreased from 3.1 to 0.1–0.3 g/100 ml following ultracentrifugation. No detectable phosphate was lost from the serum or fluid on ultracentrifugation. The initial level in serum was too low to register the predicted differences due to protein binding.

These data provide two important points of information. First, the high phosphate concentration in Fl_{SA} cannot be attributed to a mineral phase. Second, it appears that calcium was bound to macromolecular components of the cartilage fluid, probably to the protein, to a greater extent per unit weight than to plasma proteins.

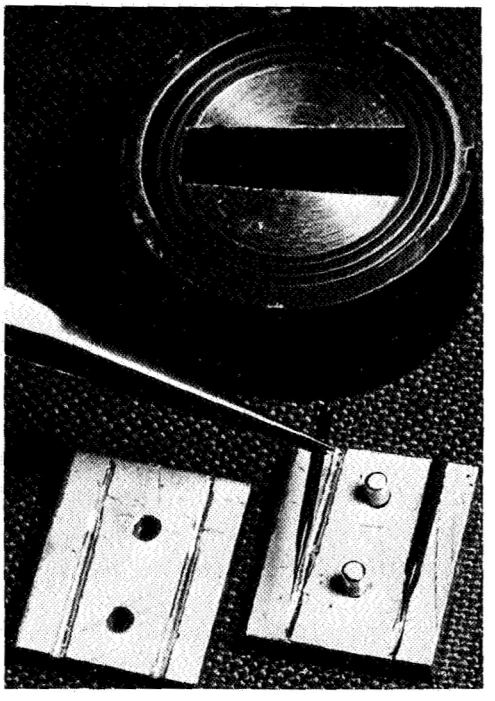


FIGURE 137. Holder for Model L ultracentrifuge and micropipet in position.

TABLE XXVII

ANALYSES OF SUPERNATANT FRACTIONS OF RACHITIC RAT SERUM AND HYPERTROPHIC CELL CARTILAGE FLUID (Fl_{SA})[a]

	Serum	Fl_{SA}
Number of animals	10	10
Pi_{TCA} (mmoles/l)	0.65 ± 0.1	1.7 ± 0.1
Pi_{TCA} in supernatant (mmoles/l)	0.60 ± 0.1	1.7 ± 0.1
Nonprotein-bound calcium × nonprotein-bound Pi_{TCA} ($M \times 10^{-7}$)	0.91	2.1

[a] Acid-soluble phosphate, Pi_{TCA}; supernatant fractions after ultracentrifugation.

URIST: That is very interesting. Dr. McLean, can you interpret these findings for us?

MCLEAN: I am afraid not.

URIST: Dr. Howell, how do you interpret the results of these analyses? The chemical composition of the fluid obtained by micropuncture,

which we will call "extracellular fluid," is quite different from an ultrafiltrate of plasma, in chemical composition.

HOWELL: Yes.

PRITCHARD: Is it extracellular fluid?

URIST: It is "extracellular fluid" of cartilage.

PRITCHARD: If you are getting some of the fluid from the cell cytoplasm, would that not have a lot of phosphate in it?

HOWELL: That problem has been a major concern and was partly the reason for measuring chloride, nucleotide, and protein.

FREMONT-SMITH: The high water content of the fluid as compared with plasma would argue very strongly against any significant amount being intracellular fluid, because there you would expect a higher protein content and less water.

HOWELL: Yes.

FREMONT-SMITH: You did not use the freezing point or osmotic pressure?

HOWELL: No.

PECK: Another possibility might be to measure the potassium concentration.

HOWELL: Potassium measurements would be ideal but difficult for us at the present time. Electrometric chloride titration has been a rather easy ancillary technique to have in operation, concurrent with the other methods described.

PRITCHARD: Did you get a lot of contaminated specimens that you did not use?

HOWELL: No; we discard perhaps 5 percent of data based on clear-cut errors of technique, particularly accidental blood aspiration. The hypertrophic cell cartilage is quite transparent and the bony borders readily visualized. Fortuitous puncture of a few cells with a micropipet could hardly account for our findings.

BAUER: Have you made any tracer studies by injecting ^{32}P intravenously?

HOWELL: Again, the volume is too small. You cannot compute the amount of radioactivity available. You could use that if you had an *in vitro* incubation, but you could never label it heavily enough *in vivo.*

BÉLANGER: So the conclusion is that this particular region of the cartilage is capable of concentrating soluble phosphate, is that it?

HOWELL: With regard to the high Fl_{SA}/serum ratio of Pi_{TCA}, it remains to be demonstrated whether this represents a physiologic mechanism to elevate inorganic phosphate for calcification or whether it merely reflects slow catabolism of the increased mass of senescent hypertrophic cartilage cells characteristic of rickets. As for the former possibility, the local action of a phosphate transferase (ref. 200) on nucleotides such as ATP might have regulatory effects on calcification,

and the elevated inorganic phosphate may be a reflection of these processes.

In any event, we hope to distinguish between hypothetical mechanisms for regulation of mineral ion transfers in the endochondral plate by further characterization of these microscopic fluid phases, one (Fl_{SA}) drawn from a site which potentially or actually calcifies, and the other (Fl_{SB}) drawn from an adjacent noncalcifying site.

BÉLANGER: Also, we know that in this particular area, this chondroitin sulfate outside the cell does concentrate calcium which is in chelated form. This we demonstrated histochemically a few years ago by putting demineralized, fixed sections in radioactive calcium and doing autoradiographs (ref. 201). Dr. Migicovsky, in his laboratory, has extracted the chondroitin sulfate from this mass of cartilage and has shown that it could chelate calcium.

MCLEAN: What is the ion product in this?

HOWELL: One may compute an approximate nonprotein-bound calcium × phosphate product. For these cartilage fluid samples from untreated severely rachitic animals, the average product was about 2.1×10^{-7} *M*. (See table XXVII.)

FREMONT-SMITH: Are there any data on pH?

HOWELL: I would rather not be quoted on figures yet, but we have made many measurements and it appears to range from 6.9 to 7.1 in the untreated rachitic cartilage on these samples that can be isolated under oil.

HOLTZER: Is there any comparison with vitreous humor in this kind of stuff?

FREMONT-SMITH: The protein is a good deal higher. In the vitreous humor, the protein is very unlike aqueous humor. Aqueous humor is very low, down to a level of about 40 or 50 milligrams, I think, and this is up to 2 grams; there is a major difference in that respect.

URIST: Aqueous humor is also low in total phosphorus—only about 1 to 2 mg/100 ml.

RAISZ: Do you think it is inorganic phosphate or not? I ask this because there could be a difference in nucleotide content. Was it possible to analyze for any soluble organic phosphates?

HOWELL: We have run ATP and guanosine triphosphate through our analytic system and it does not register as a trichloroacetic acid-soluble phosphate. However, histidine-nitrogen-linked phosphate would be liberated under pH 7. We are unable to distinguish acid-labile from inorganic phosphate in cartilage fluid.

URIST: Let us return to the subject of cellular differentiation and interrogate the experts on induction systems. Perhaps we can find areas of some general agreement. Induction takes place with inter-

action of cells. There are two groups of cells in every induction system: inducing cells and responding cells. In both groups, the cells are proliferating and not resting. We are not able to tell these cells apart or classify them or give them a specific name. Dr. Saxén, can you challenge these simple statements about induction systems?

SAXÉN: I may just add one thing. When you said that there is an inducing cell and a responding cell—we know some model systems where this interaction seems to be mutual. That is, the responding cells are somehow inducing or interacting with the so-called inducing cells. This is seen in the development of the central nervous system, at least.

FREMONT-SMITH: A sort of mutual feedback?

SAXÉN: Yes. They can do both.

URIST: They can function either as responding or inducing cells, is that the idea?

SAXÉN: I would not put it quite that way. Let us say with regard to the inducing cells, their development is somehow dependent on the presence of the responding cells.

HOLTZER: I think "reciprocal" would be a good word, and I do not want to get into semantics, but I must quickly point out that there is vitamin A. Does one need a cell? Vitamin A is the nicest induction system I know. The other point is the homotypic interaction mentioned yesterday, where there is crosstalk between like cells. So, with these provisos, I am sure you can go ahead because it will not reduce the impact, but, I think finally, if we are ever going to get to a model of induction, which I assume we are leading to, we are going to have to contend with a passing remark by Dr. Arnaud yesterday, and that is, "What is a hormone?" I just anticipate this right from the start.

URIST: Yes; but let us first consider the development of the cells that the hormones are going to act upon.

YOUNG: Dr. Urist, some people, such as myself, have taken a general view that all changes in cell specialization probably involve the same intracellular mechanism, and consequently the shift of specialization within normal growing bone would not be different in mechanism from the shift that you might observe in your experimental situation. In that regard, do you agree or disagree that any change in cell specialization represents an induction system?

URIST: I think I would agree that induction systems bring about differentiation of specialized cells.

YOUNG: So that if one of the precursor cells in bone becomes an osteoblast, that is also an induction?

YOUNG: I think it is important to emphasize that the complexity of this reaction is probably within the cell and involves the selective activation and repression of integrated groups of genes, and that there is no

reason to think that this mechanism differs either prenatally or postnatally or even in unicellular or multicellular organisms or *in vitro* or *in vivo*.

URIST: We will try to discuss the question of genetic mechanisms later. The question is, assuming that there is an inducing cell or tissue, does an inducer exist? The literature defines inducers as many different kinds of things, such as small protein molecules, changes in oxygen saturation, CO_2 tension, mechanical factors, pressure or stress and strain. Thus, an inducer may be something from the outside which is a chemical entity, possibly a lipoprotein, a mucoprotein, or a protein-protein complex; it may be a change in intercellular or intracellular oxygen saturation and CO_2 tension.

If we define an inducer as something which may have any one of these forms, then it is reasonable to assume that inducers do exist.

HOLTZER: You must mention inorganic ions to make the list reasonably complete.

URIST: Let us add inorganic ions, endocrine factors, metabolites, antimetabolites, and activators. In the broadest sense a hormone is an activator.

PRITCHARD: I thought it was a chemical messenger.

FREMONT-SMITH: "I arouse to action: *hormao*." So an activator I think is all right. It is a messenger which activates.

URIST: The word "activator" is used by molecular biologists who are changing the language of cell physiology to make it broad in perspective and applicable to systems *in vitro* (ref. 202).

PRITCHARD: If you now change from discussing what goes from one cell to another to what happens when it gets there, which is a different concept——

URIST: If we define an inducer as something as broad in its nature as the factors in all of these categories, could everyone agree that an inducer exists? Do you have any objection to that?

PRITCHARD: I do not think we need to be too specific. Some vehicle for transmission of information from one cell to another is all that is necessary.

URIST: What would the vehicle convey?

PRITCHARD: Some vehicle for transmitting information from one cell to another.

URIST: How would you define the word "vehicle"?

PRITCHARD: Some means of getting a message across. It might be electrical, physical, chemical.

URIST: Let us add the effects of electrical stimuli. Is there anything else you can add?

PRITCHARD: Any other channel of communication anybody can think of.

URIST: Let us add "unknown factors."

YOUNG: I would like to suggest that most inductive reactions—that is, changes in cell specialization—may be traced back to a change in the microenvironment of the reacting cell (refs. 111, 203, and 204). Where we have some information on what seem to be the critical aspects within this admittedly complex microenvironment, it appears that they fall in this sort of category, where we see that the critical agent has very little inherent specificity or complexity in itself, again emphasizing that the complexity is in the reacting system.

URIST: I have drawn a diagram of a cell. The cell membrane appears as a broken line to show that there is communication with the outside world; the same applies to the nuclear membrane.

FREMONT-SMITH: I think nature broke it for you, but I am glad that you copied nature.

URIST: Diagrammatically, we are dealing with an open system.

FREMONT-SMITH: You mean a partly closed system.

URIST: Yes; assuming that inducers exist, defining the inducer as broadly as possible, where is it located? Is the inducer intracellular or is it extracellular? Since induction systems represent systems in which there is interaction of cells, can we assume that the inducer is cellbound and transported by cells?

PRITCHARD: What is the inducing cell and what is the responding cell?

URIST: The progeny of a young chondrocyte in the germinal layer of the epiphyseal cartilage are inducing cells. The evidence is that if you transplant a piece of cartilage that has germinal layer cells, these cells do not go on and make more cartilage but they produce chondrolysis, interact with the ingrowing cells of the host bed (the mesenchymal or responding cells), and where this interaction occurs you see an induction system for bone formation.

HOLTZER: But is it not fair to point out that in fact you do not know the source of that bone cell? There is no rigorous demonstration that this particular osteocyte can trace its lineage back to either what you could call the inducing system or the responding system. It is there.

URIST: You are correct. We cannot say whether or not a cell came from the host bed or whether it came from the young modulated chondrocyte. What I observed was that there was: (1) cell proliferation, (2) mixing of cells from two sources, and (3) differentiation of bone in excavation chambers in which the cells are swarming.

OWEN: Marijke Holtrop (ref. 205) has shown that at least some of them come from the cartilage cells.

URIST: The modulated cartilage cells can modulate and become osteoblasts.

RAISZ: And also host cells became osteoblasts in the same preparation.

OWEN: The cartilage cells were labeled before transplanting the piece of cartilage and after transplantation labeled osteoblasts and osteocytes were found.

URIST: We also labeled the cartilage cells and found a few labeled osteocytes. Our observations of callus and articular cartilage suggest that a very small number of osteocytes may be linear descendants of modulated labeled chondrocytes and that modulated chondrocytes may act under special circumstances as responding as well as inducing cells. The source of the induced cell is immaterial. The important thing is whether the pathway of the cell has been altered by interaction with other cells in an excavation chamber. The following five tables present data on transplants of articular cartilage labeled with ^{3}H-thymidine for nuclei and ^{3}H-glycine for matrix. The matrix label enables one to distinguish transplanted cartilage from new cartilage formed after transplantation.

TABLE XXVIII

FATE OF ^{3}H-THYMIDINE LABELED CHONDROCYTES AFTER TRANSPLANTATION OF ARTICULAR CARTILAGE INTO ANTERIOR CHAMBER

Number of implants	Days in anterior chamber	Cells counted	Cells labeled		Grain count > 50 or < 50		Percent of labeled cells in specific cell type		
			Total	Percent			Round	*Hypertrophic*	*Squamous*
1	1	5107	142	2.78	>		100	0	0
						<	98	2	0
1	3	1639	22	1.34	>		86	0	14
						<	100	0	0
1	4	2043	3	.15	>		100	0	0
						<	100	0	0
1	5	3159	10	.32	>				
						<	90	0	10
1	6	1596	14	.88	>		0	100	0
						<	87.5	0	12.5
1	8	1190	6	.50	>		66.6	0	33.3
						<	100	0	0
4	18	6241	32	.43	>				
						<	100	0	0
5	28	2912	6	.21	>		100	0	0
						<	100	0	0

Table XXVIII illustrates the percentage of labeled cells in transplants of articular cartilage at various intervals of from 1 to 28 days. The labeled cells were chiefly round chondrocytes of the germinal layer at all intervals after transplantation. This suggests that, following transplantation, the cells reduce the rate of further development of

cartilage and assume the pathway of development of cells for bone induction.

Table XXIX shows the relative constant proportion of round (zone B) to hypertrophic to squamous (zone A) cells in double-labeled nuclei and matrix transplants before and after bone induction. This observation affirms the fact that transplantation suppresses further proliferation of articular cartilage.

TABLE XXIX

FATE OF CHONDROCYTES WITH ^{3}H-THYMIDINE LABELED NUCLEI AND ^{3}H-GLYCINE LABELED MATRIX IN ARTICULAR CARTILAGE TRANSPLANTS

Number of implants	Days in anterior chamber	Cells counted	Cells labeled		Percent of labeled cells in specific cell type		
			Total	Percent	Round	Hypertrophic	Squamous
7	14	26 267	331	1.26	93	4.8	2.2
10	28	20 177	428	2.12	89.7	9.3	1
14	35	25 518	336	1.32	91.4	3.8	4.8

Table XXX lists the progressive reduction in the percentage of labeled cells, presumably modulated chondrocytes, in excavation chambers produced by the host in transplants of articular cartilage. The decrease in the number of cells with high grain counts and the increase in the number of cells with low grain counts indicate that chondrocytes modulate and undergo mitotic division inside the excavation chambers.

TABLE XXX

FATE OF EXCAVATION CHAMBER CELLS OF ARTICULAR CARTILAGE LABELED WITH ^{3}H-THYMIDINE AND ^{3}H-GLYCINE BEFORE AND AFTER BONE INDUCTION

Number of implants	Days in anterior chamber	Cells counted		Grains counted		
		Total	Percent	> 50	≤ 50	Average
7	14	1 130	7.7	1	87	10.6
10	28	16 684	2.4	42	351	14.3
14	35	24 770	1.1	35	240	10.7

Table XXXI summarizes counts of labeled bone cells produced from transplants of double-labeled articular cartilage cells. The percentage

of labeled osteoblasts and osteocytes was relatively low, but the average grain counts were at least 10 percent of maximum. These data suggest that a small, indeterminate number of the bone cells are linear descendants of modulated chondrocytes.

TABLE XXXI

FATE OF CELLS OF ARTICULAR CARTILAGE TRANSPLANTS LABELED WITH ^{3}H-THYMIDINE AND ^{3}H-GLYCINE, OUTSIDE OF EXCAVATION CHAMBERS OR AREAS OF BONE INDUCTION

Number of implants	Days in anterior chamber	Osteoblasts			Osteocytes			
		Total counted	Percent labeled	Average grain count	Total counted	Number labeled[a]		
						Total	Percent	Average grain count
7	14	0	0	0	0	0	0	0
10	28	6 788	.13	7.3	5 239	34	.6	10.5
14	35	13 050	.05	15.2	10 866	44	.4	9.9

[a] All labeled cells had 50 grains.

Table XXXII summarizes grain counts of chondrocytes in articular cartilage transplants. These cells always had high grain counts, gave the appearance of cartilage cells transforming directly into bone cells, and consisted mainly of inclusions of unabsorbed cartilage in the middle of an island of bone tissue.

TABLE XXXII

FATE OF CELLS OF ARTICULAR CARTILAGE TRANSPLANTS LABELED WITH ^{3}H-THYMIDINE AND ^{3}H-GLYCINE

Number of implants	Days in anterior chamber	Chondroid		
		Total counted	Number labeled[a]	Percent
7	14	11 631	43	0.37
10	28	3 990	37	.93
14	35	1 588	13	.82

[a] All labeled cells had > 50 grains.

YOUNG: If we agree that the mechanism is the same, it does not matter for the purposes of this discussion which cells were induced.

URIST: But the point is that the progeny of these cells are not inducing the old adults. They are inducing the young, new generations

TABLE XXXIII

Induction Systems for Osteogenesis, Chondrogenesis, and Myelogenesis in Articular Cartilage Transplants

Time, weeks	Inducing cell type	Responding cell type	Hypothetical changes locally in microenvironment	Responding cell	Induced cell
0	Germinal chondrocyte zone B ( )				
	↓				
2	Resorption of cartilage matrix 1st mitotic division	+ Perivascular connective tissue cell of host, several mitotic divisions			
	↓	↓			
3	Rapidly dividing young connective tissue cells in excavation chambers	+ Perivascular connective tissue cell of host, several mitotic divisions	⇄ Low CO_2 tension, high O_2 saturation, compaction	⇄ Mitotic division and further increase in population of responding cell, young fibrous connective tissue cells	
	↓	↓	⇄	↓	
4	Rapidly dividing young connective tissue cells in excavation chambers	Perivascular connective tissue cell of host, several mitotic divisions	⇄	⇄ Mitotic division and further increase in population of responding cell, young fibrous connective tissue cells	Osteoprogenitor cell, osteoblasts, osteocytes
	↓	↓	High CO_2 tension, low O_2 saturation, compaction	↓	
4	Rapidly dividing young connective tissue cells in excavation chambers	Perivascular connective tissue cell of host, several mitotic divisions	⇄	⇄ Mitotic division and further increase in population of responding cell, young fibrous connective tissue cells	→ New chondroprogenitor cell, chondrocytes, chondroblasts
	↓	↓		↓	
4	Rapidly dividing young connective tissue cells in excavation chambers	Perivascular connective tissue cell of host, several mitotic divisions	⇄ Normal O_2 tension Low CO_2 saturation, low pressure	⇄ Mitotic division and further increase in population of responding cell, young fibrous connective tissue cells	→ Fibroprogenitor cells, fibroblasts, fibrous tissue
	↓	↓		↓	
6	Rapidly dividing young connective tissue cells in excavation chambers	Perivascular connective tissue cell of host, several mitotic divisions	High O_2 saturation, low CO_2 saturation, low pressure	→ Mitotic division and further increase in population of responding cell, young fibrous connective tissue cells	→ Hemopoietic stem cell, hematoblasts, bone marrow

of cells, and the process of bone induction may take place during a period of one or more mitotic divisions. Table XXXIII is a schematic representation of the time sequence and the hypothetical changes in the microenvironment of a transplant of articular cartilage, associated with the appearance and development of new fibrous tissue, bone, cartilage, and bone marrow. Similar to embryonic induction systems, for all kinds of organs, bone-induction systems produce many different kinds of cells concurrently or in a sequence in addition to osteoblasts and osteocytes.

PRITCHARD: It might be worthwhile to drop the word "induction" altogether.

URIST: It might be worth considering all the evidence in its favor first.

PRITCHARD: You have two populations merging.

URIST: Yes.

PRITCHARD: As a result, certain things happen which lead to bone formation. Some of the cells of that mixed population apparently become osteoblasts. That is all you have really demonstrated. The rest is hypothesis.

URIST: You cannot mean all we have demonstrated. You may mean what we have demonstrated.

PRITCHARD: All—in this system.

URIST: Before we discard the word "induction," let us see if we can use it; if not, we will be glad to dispose of it.

PRITCHARD: It could be a handicap—the language.

FREMONT-SMITH: If the word "induction" does give you a theory that provides experiments to show you whether or not the theory is correct. There is a great advantage in theory, because it induces experiments, and we are looking for the induction of certain experiments as a result of theoretic development.

URIST: We are perfectly willing to throw out that word and the whole concept of induction, but before we do that we must have a reason.

PRITCHARD: Well, the fact that there is so much confusion and so little evidence as to what these transmitting agents are——

URIST: Some of the confusion is in the mind of the possessor, and some of it is not. Let us go on, and perhaps we can see more evidence.

PRITCHARD: All right.

SAXÉN: I have the very same impression, that people usually do not like the words, "induction," "inducer." It somehow seems to irritate people.

FREMONT-SMITH: That means it makes them uncomfortable.

SAXÉN: That may be. Some years ago, in a meeting such as this, somebody suggested that instead of speaking of induction we might

say that something provides conditions necessary for a certain type of differentiation situation. It is easier to use the word "induction," but we really do not always refer to such specific approaches, and all this that has been listed might just be a kind of proper environment, proper condition for a subsequent development.

URIST: Yes; the inductor may be a highly complex list of chemical, physical, electrical, and mechanical influences. As a matter of fact, it could be an entity as complex as life itself.

NICHOLS: How rigorous is the evidence that no cells can transmute from one type to another without cell division?

URIST: In bone-induction systems, cell division precedes differentiation of cells into osteoblasts.

NICHOLS: I asked you, How rigorous is the evidence? In a sense, Marijke Holtrop's experiments (ref. 206) suggest that cell division is not a prerequisite for the process.

URIST: I am uncertain about whether mitosis is always a prerequisite.

NICHOLS: The question is, Must there be cell division in order to get specialization?

URIST: When you say "must," you confront us with the problem of interpreting exceptions to the rule that mitosis precedes differentiation of osteoprogenitor cells.

NICHOLS: That is what I meant.

URIST: When you say "must," you raise a different kind of question; the plain fact, however, is that preparation for mitosis is the basis of uptake of tritiated thymidine, and the labeling of osteoprogenitor cells.

HOLTZER: I would like to hear a little more about these experiments. I do not know they work at all. Has somebody labeled a cartilage cell and observed it transforming into an osteoblast without undergoing a cell division?

OWEN: I feel that the work is not conclusive on this question because, as far as I know, grain-count studies have not been done, although they may have been done recently. In this case, therefore, it is possible for a labeled cartilage cell to have gone through one or more divisions before it is seen as a labeled osteoblast, and so forth.

HOLTZER: This is an important point.

NICHOLS: I think some of this depends on how we are going to define "induction."

URIST: We are going to try to define "induction" on the basis of experiments upon material in which there is always clear-cut evidence of cell division before there is cellular differentiation. The occurrence of bone formation in small amounts by an ancillary process is an exceptional situation and does not permit us to dispose of the occurrence

of bone formation by the process of induction of rapidly dividing cells in large numbers in a typical situation.

BÉLANGER: What else do you see?

HOLTZER: Are you going to accept "osteoblast" and "osteoclast" as "induction"?

YOUNG: My opinion is that basically the intracellular rearrangements must be the same. The cell is not set up to have six or seven different ways of changing its specialization depending on the experimental situation.

NICHOLS: Some of us do not know that that happens.

HOLTZER: Actually, I agree with you and I hope you are right, but in point of fact, nobody has really rigorously eliminated the possibility of some kind of other intracellular rearrangement.

URIST: Are you raising the question of whether or not one specialized cell form can turn into another specialized cell form without either mitotic division or modulation? In the older literature, this was termed "metaplasia." We can discuss that subject after we examine the role of mitotic division in a typical bone-induction system.

BÉLANGER: May I say just one thing? This is with reference to the observations of Dr. Holtrop (ref. 205). I have seen her material and I have read her thesis. The only evidence, to my mind, is that in some of these empty lacunae of the hypertrophic cartilage, there are cells which look like osteoblasts and which are labeled. Now, we know that in this particular area the cartilage cells die. Is it not possible that as they die in large numbers and they have labeled nuclei, the precursors of the osteoblasts that are just coming in might utilize this material to make their own DNA? Is this not possible?

OWEN: I think it is very unlikely, although possible.

BÉLANGER: Even in a system such as this, where there is a concentration locally? This is in culture, mind you; this is not *in vivo*. I just want to make the point that just interpreting such important phenomena on purely morphologic data is dangerous.

URIST: Here we bring into evidence the results of an experiment that we know about only partially.[1]

PRITCHARD: Dr. Urist, you are making a great point about mitosis, and I am wondering why you are stressing mitosis as being such an important aspect of your theory. Is it essential?

URIST: I have not given my theory yet.

PRITCHARD: You are leading up to it.

URIST: A definition of the term "induction" is a mechanism of

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[1] Holtrop (ref. 206) concluded: "Cartilage cells probably first differentiate into connective tissue cells, periosteum or perichondrium, and then bone afterwards."

cellular differentiation that occurs from the interaction of two cells, an inducing and a responding cell, as a result of which the latter follows a pathway of development it otherwise would not have exhibited. I have not proposed a theory, but I assume that mitosis occurs before, during, or after induction. In every bone-induction system I have seen there is a preliminary stage of cellular proliferation. The inducing and responding cells are the progeny of two different preexisting groups of cells.

HOLTZER: I do not think there is any question about this. If one goes through the literature on induction, mitosis is certainly there. In the sense of a purist, I was a little shocked at the unequivocal usage of this particular experiment as evidence against mitosis.

OWEN: No; I think you have misunderstood what has been said.

HOLTZER: I would say there is not a single induction experiment that, to my knowledge at least, does not involve mitotic activity.

PRITCHARD: Do you mean mitotic activity immediately preceding induction? I have observed bone and cartilage formation in a repaired tendon several weeks after the burst of mitotic activity which follows tenotomy. I had the impression that the tendon cells became transformed into bone and cartilage cells without any mitosis in between.

URIST: Have you described tendon cells turning directly into bone cells?

PRITCHARD: It was well described by Buck (ref. 207) in 1953. Turkey tendons ossify, I believe, without mitosis. The formation of bone does not necessarily imply the intervention of some mysterious induction system, nor is it necessarily preceded by mitotic activity. In the normal development of the skeleton, a lot of tendon is converted into bone without the cells undergoing mitotic division. Presumably, something induces the change, but whatever it is it does not have to cause the cells to divide first.

URIST: You have cartilage cells in tendon insertions that may be inducing cells that are partly induced in the direction of osteogenesis.[2] Ossification of the tendon is associated with an interaction of cells, and it is a process that takes place like an infectious wave that moves through the tendon. There are systems in which it would appear as though one specialized cell form transforms directly into a bone cell

[2] Buck (ref. 207) described multiplication of new cells of the *peritenonium internum* inside the cut ends of the tendon, and noted that it was unlikely that mature tendon cells undergo mitosis. He found bone at the junction of the new cells with the old stumps of tendon, but not until about the third month after the tenotomy. Buck thought that the new bone was closely associated with cartilage-like cells in the area of repair, and wrote that cartilage and bone may represent metaplasia of fibroblasts in the regenerating parts (not the previously existing parts) of the stumps of the tendon.

without modulation. This occurs in the deer's antler, but the tissue is referred to as "chondroid," because it looks like cartilage but is not true cartilage. The way it happens is something like in birds in which the cartilage cells appear to transform into bone cells. Nevertheless, in the process, cells proliferate along an edge, blood vessels sprout, periosteum-like and endosteum-like tissues are involved, and many undifferentiated cells are recruited all along the way to differentiate first into osteoblasts and then into osteocytes. If a specialized cell appears subjectively to transform directly into an osteocyte, it is necessary to consider its past history and possible effects as an inducer as well as a transformer.

PRITCHARD: Well, we have been studying tendon repair for the past 2 years, and we have been looking at tendon and ligament attachments to bone. Transitions from tendon cells to bone cells are perfectly clear.

URIST: Please present the evidence for this view.

PRITCHARD: I thought it was very well known.

URIST: The process of ossification of tendon may give one the impression that tendon cells transform into bone cells, and this could be a side issue. The mainstream of cell differentiation comes from responding cells around ingrowing capillary sprouts. Transforming or modulating tendon cells may be inducers.

SAXÉN: I agree completely with what Dr. Holtzer said. It is very easy to design an experiment to study mitosis. You combine the responding tissue with an inducer, and the control with another tissue which does not lead to morphogenesis in the responding cell. One of the very first changes in the induced cells is an increased uptake of thymidine.

URIST: Yes.

SAXÉN: Whereas in the other case, where the cells survive, you do not see this.

HOLTZER: Just for the record, for the past 2 years we have been studying the necessity for mitosis in the two induction systems that we have been working with for many years.

FREMONT-SMITH: You found it necessary?

HOLTZER: Yes.

URIST: To observe the phase of mitosis in an induction system, a cell-labeling technique is often helpful.

PRITCHARD: How do you know I have not done this?

URIST: I have read the articles by Pritchard and associates on the cells of the blastema in healing fractures in vertebrates in unlabeled histologic section.

PRITCHARD: That is not all I have written.

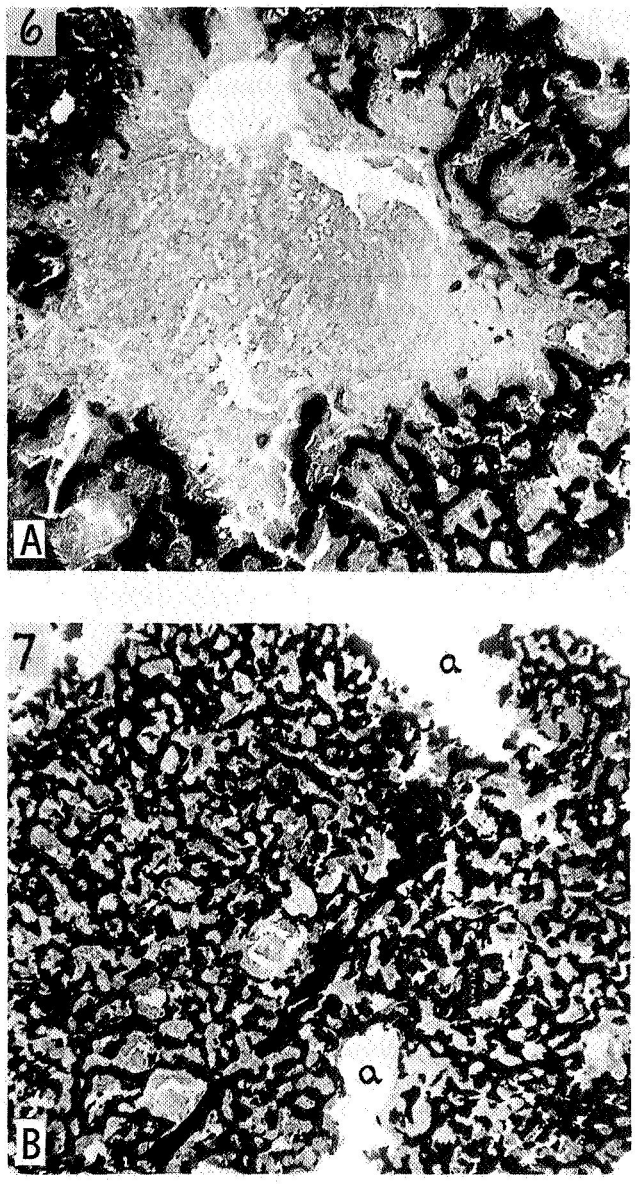

FIGURE 138. Photomicrographs of undecalcified femoral marrow of pigeons. (*a*) Section from a pigeon with a 6-millimeter follicle in ovary; the pale-staining central core of hemopoiesis marrow is in sharp contrast to the black trabeculae of medullary bone. (*b*) Section from a pigeon with a 20-millimeter follicle in ovary; the bone has permeated the entire marrow. The gaps marked *a* are the result of penetration of the marrow by the dental drill used to remove the dense cortical bone. Fixation: 10 percent neutral formalin; von Kossa stain. 35×. [From Bloom et al., ref. 175; reprinted with permission of the publisher.]

MCLEAN: Would you like a clear-cut demonstration of this induction process?

URIST: Yes; we need some tangible material to discuss the subject of mitosis.

MCLEAN: This is the system of endosteal bone formation, medullary bone formation, in the pigeon, without any external influence. This is what the pigeon does.

In figure 138(*a*), bone is growing out into the marrow cavity. This reaction is initiated in the endosteum, and it proceeds in a wave out into the marrow cavity until, as you see in figure 138(*b*), it has filled the marrow cavity with new bone. This is the gross phenomenon.

Figure 139 shows what is going on in this process. At the bottom there is a small spicule of bone which has formed at the endosteum, and the wave proceeding up into the marrow with abundant mitoses can be seen. The cells, the reticular cells, are in the process of being transformed into bone cells. This work was done by Bloom et al. (ref. 175), and it is as clear a demonstration of what we are talking about as I know of, because we can see the wave as it progresses into the marrow, and the marrow reticular cells are being drawn into the wave by what we are calling induction. Do you agree with that?

URIST: Yes. And there is abundant cell division and proliferation as this process goes on.

MCLEAN: It is full of it.

PRITCHARD: Can I describe my induction system? I think it is fundamental and should not be discussed as being wrong because it was done by a different histologic method.

URIST: If you have evidence, it will not be dismissed or undervalued; please present it.

PRITCHARD: This is the induction system that we have been working on. If you take the tendo Achilles of a rat, young or old, cut it cleanly and let it repair by itself, in 40 to 70 days you will invariably find that bone has developed in the tendon, not only in the new tendon, but also in the old tendon ends. There is a long lag period.

URIST: How long?

PRITCHARD: Up to 40 days after operation. In the first few days, there is a lot of mitotic activity. After that, the cells are engaged in collagen production, and during the maturation of the new tendon you do not find mitotic figures.

URIST: How do you define the term "maturation of the tendon"?

PRITCHARD: During the maturation process, the collagen fibers become thicker and more densely packed and the cells get flatter—the sort of thing you would expect in any repair.

URIST: What is the origin of the cells?

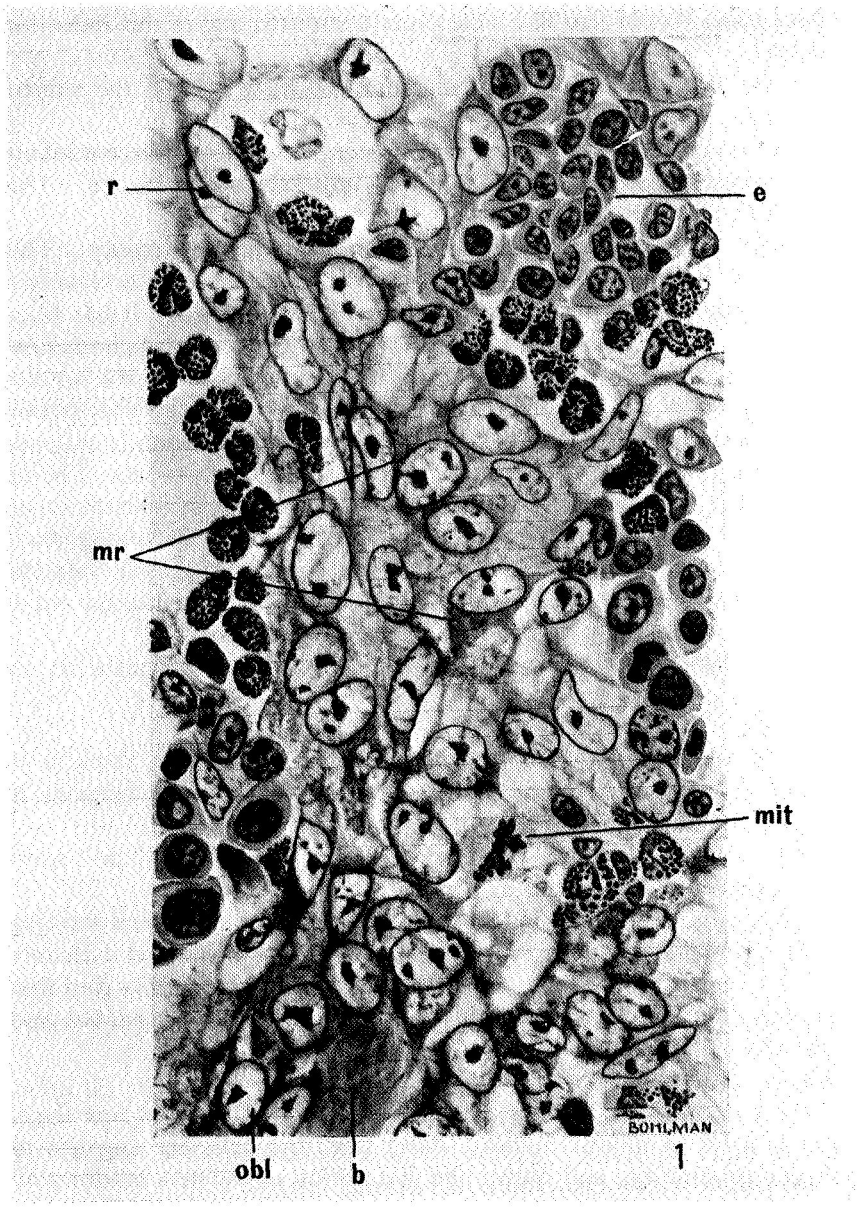

FIGURE 139. Photomicrograph of section of bone marrow from pigeon with 6-millimeter follicle in ovary, showing changes in reticular cells (r) and osteoblasts (obl), ahead of the advancing trabecula of bone (b); mobilizing reticular cells (mr); mitosis of reticular cell (mit); erythropoiesis (e). Zenker-formol fixation; HEA stain. 1650×. [From Bloom et al., ref. 175; reprinted by permission of the publisher.]

PRITCHARD: These cells come largely from the epitenon, but some come from the tendon ends, because one sees mitotic figures in the tendon ends. If the operation is done roughly, they also come from the paratenon.

URIST: How many generations of cells or cell divisions were derived from cells of the paratenon?

PRITCHARD: From the early——

URIST: How many cell divisions occurred in the time of your experiment? You cut the tendon?

PRITCHARD: Yes.

URIST: There was an ingrowth of cells from the tendon sheath. How many cell divisions occurred in 40 days?

PRITCHARD: There probably were many to begin with. In any repair process, mitotic activity is concentrated in the first few days; after that, the formation of collagen is the predominant feature. I think this has been displayed so often in repair processes that I do not have to prove it. After this time, when, to all intents and purposes, you have a tendon that is mature, you begin to see these changes in the tendon without any sign of mitosis.

FREMONT-SMITH: Without any further signs of mitosis.

PRITCHARD: Oh, all right, but any tissue in the body has had a long history of mitotic activity.

FREMONT-SMITH: But the mitosis that took place here took place as a result of the trauma, and not without any further mitosis.

PRITCHARD: Without any further mitosis these cells changed their form, and their matrix became calcified.

URIST: Does the new tendon come from new fibrous connective tissue and is this new tissue fibrous tissue or tendon?

PRITCHARD: It is somewhere between fibrous connective tissue and tendon. You know tendon never repairs perfectly.

URIST: The end product of repair of a cut tendon is a scar or irregular meshwork of fibrous tissue; the fibers are not organized in parallel bundles like the original tendon.

PRITCHARD: A repaired tendon with the fibers parallel is different from a scar with irregular fibers all over the place. It is new tissue that has matured and it is getting toward the tendon state, and if it is left for a year it gets even more like normal tendon.

HOLTZER: I wonder if I might try to resolve this. We might want to go on to something a little more interesting. Clearly, cells do divide and they have an evolution. I think this is the point. You take a cartilage cell, and after a while keratosulfate, for example, comes into the system. This is part of the normal repertoire of an aging cartilage cell. This is one of the things about aging. A cell gradually—very often——

PRITCHARD: I think this is well known, too. All I am suggesting is that a cell which has spent part of its life as a tendon cell can become a bone cell without having to go back to square 1 and revert.

Figure 140 shows a repaired tendon and demonstrates the transition zone—the tidemark where tendon stops and bone begins. There are lines and lines of cells going from the tendon into the bone without mitosis.

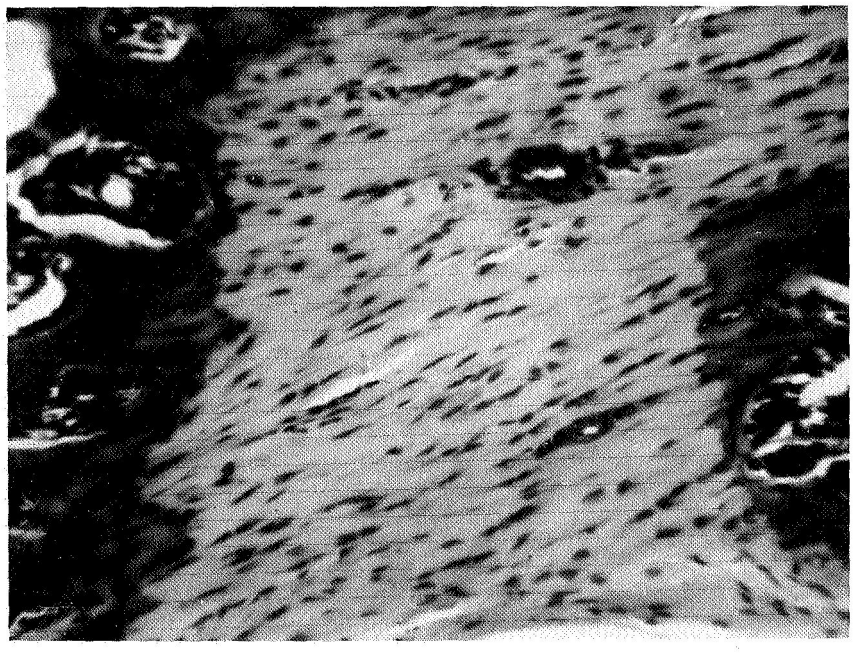

FIGURE 140. Photomicrograph of patches of ectopic bone in repaired tendo Achilles of the rat 100 days after tenotomy. 220×.

FREMONT-SMITH: At that time.

PRITCHARD: Well, yes; at that time.

FREMONT-SMITH: But they did have new mitoses just a few weeks before. So the issue here, as I see it, and I really think it is quite clear, is: How long after mitosis can a cell produce bone and not be considered related to the previous mitosis?

PRITCHARD: Plus one other point. While it is in this postmitotic state, can it produce collagen before it goes on to finish the job of making bone? I think it can.

URIST: May I attempt to explain the sequence of events that led up to this picture? Please interrupt and correct any erroneous statements. Tendon is covered with tendon sheath. After the tendon is cut, muscle contraction pulls the ends of tendon apart and creates a space.

PRITCHARD: About 1 centimeter.

URIST: That 1-centimeter space becomes filled with fibrinous clot. The tendon sheath has a capillary circulation and is the source of sprouting capillaries.

PRITCHARD: What do you mean by the tendon sheath? You and I may not be referring to the same thing.

URIST: It is either epitenon or paratenon.

PRITCHARD: Let us take the epitenon, not the paratenon.

URIST: It is a source of sprouting capillaries. First inflammation, then sprouting capillaries, and ingrowth of hundreds, of thousands, of new cells, until a bridge of connective tissue forms between the ends of the cut tendon.

PRITCHARD: I do not like this model. It is not like that, really.

URIST: You said the gap was 1 centimeter in length. What occupied the gap after a week?

PRITCHARD: The sides collapsed.

URIST: Does the original tendon sheath fill the space?

PRITCHARD: I will have to draw it, Dr. Urist, to make my point clear (fig. 141).

URIST: If the sheath collapsed, it would soon reexpand from ingrowth of new cells.

PRITCHARD: But you are guessing at something——

URIST: I have seen hundreds of tendons heal, and the ends are always enclosed in a fusiform mass of tumorlike fibrous connective tissue. The end product of repair develops from a mass of new cells.

FREMONT-SMITH: Why not let Dr. Pritchard explain his figure?

URIST: Please consider the possibility that the ingrowing mass of new cells are the responding cells of an induction system. We cannot underestimate the importance of that pathway of repair. Specialized cells such as cartilage and bone develop from the interaction of the progeny of resident and ingrowing cells in the area of the tendon gap.

PRITCHARD: You have seen two of my figures (figs. 140 and 141), and I have a story to explain; I would like to tell what really happened. I do not really want to take a lot of the audience's time, but I have been challenged on this.

After cutting the tendon, the ends with their epitenon retract, leaving a 1-centimeter retraction gap. The paratenon does not retract, but tends to collapse into the retraction gap. There is usually very little bleeding inside the empty segment of paratenon.

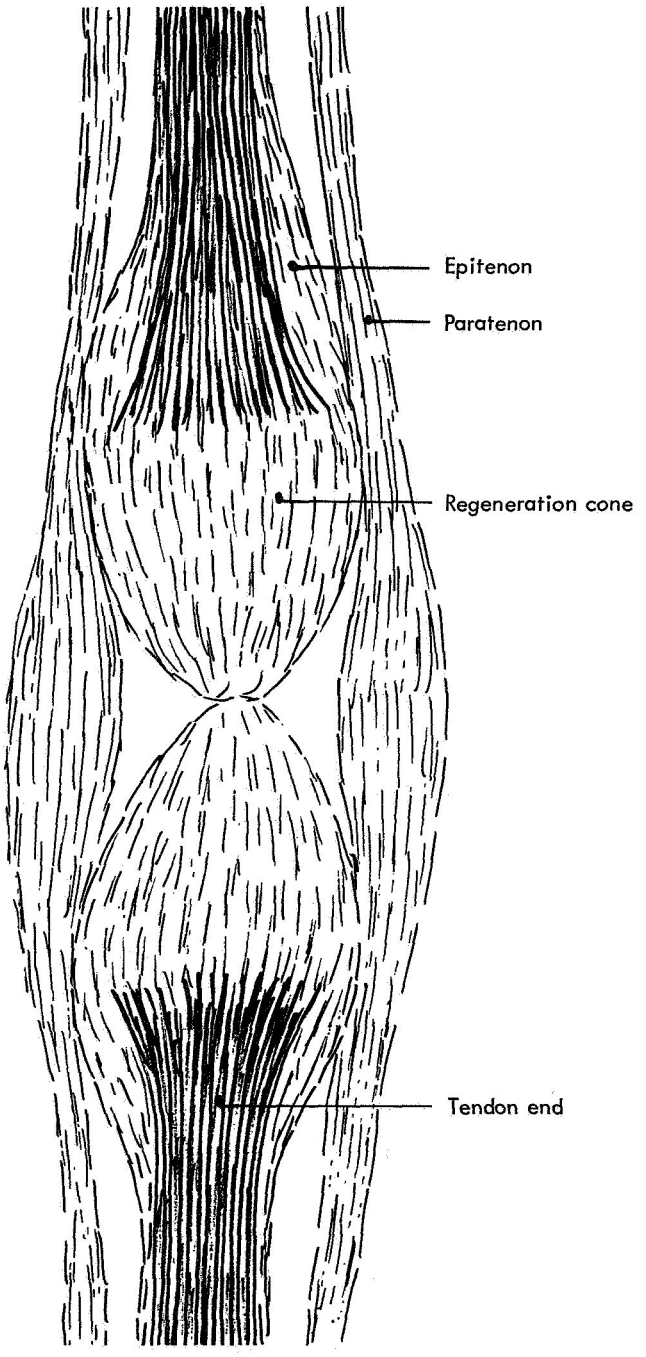

FIGURE 141. Diagram illustrating formation of regeneration cones from tendon ends after tenotomy in the rat.

The epitenon cells, during the next few days, proliferate enormously and form regeneration blastemas which push their way toward each other inside the paratenon tube. They fuse to form the basis of the new tendon which will develop to bridge the gap in the old tendon.

FREMONT-SMITH: Will there be new capillaries in there?

PRITCHARD: Oh, yes; but the capillaries do not come first.

FREMONT-SMITH: They are all together now as it is joining?

PRITCHARD: If the tendon is cut cleanly, the regeneration blastemas come from the epitenon. However, if the operation is less expertly performed, there is proliferation of a paratenon as well, and the new segment of tendon is derived from a complex of paratenon and epitenon cells. After 3 or 4 days, mitosis can be observed in the tendon ends, and cells apparently come out of the end of the tendon and join the blastema.

After 10 days, the tendon ends are grossly swollen and they are enveloped in big cones of new tissue. Now collagen formation, rather than mitosis, is the order of the day. From 30 to 40 days later, there is a functionally useful tendon again, capable of moving up and down within a new sheath. New and old tendon are so perfectly joined that the line of fusion can hardly be made out. Then, in various places, we begin to get bone and cartilage formation. The tendon is transformed into bone and cartilage. This is not confined to the new tendon but occurs in the old tendon as well.

Up to 40 days, you do not find bone or cartilage, but at 70 days you always find one or the other, or both, and along the boundary line between tendon and bone or cartilage you do not find the sort of vascular or mitotic activity you might expect if there were great cellular turnover and change. It is a quiet-looking region where tendon cells grade into bone cells.

URIST: Is it unreasonable to use the term "differentiation"? Is it incorrect to assume that the myriad of new cells in healing tendon, not the small limited number of old tendon cells, would differentiate into bone? Is it not more reasonable to assume that new cells were induced to enter a new pathway of development and specialization?

PRITCHARD: By what?

URIST: By local factors present at an earlier stage and immediately after cell division and proliferation.[3]

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[3] If the process is brought about by alterations in the regulatory genes, the time for bringing these alterations about could be immediately after mitosis during the period of high activity of the nucleolus—the organelle that receives certain species of RNA from chromatin and sends it forth to be clothed in ribosomal protein. The nucleolus is the engine of ribosomal protein synthesis and of ribosomal assemblage, according to Bonner (ref. 208).

PRITCHARD: I do not know how you can know that, especially as this phenomenon is peculiar to the tendo Achilles of the rat.

URIST: The rat is not an exception. It also happens in man.[4]

PRITCHARD: It happened to John Hunter's tendo Achilles. But surely, you are straining the concept of induction a bit far.

URIST: The cells that differentiated into bone could have developed their competence to specialize as osteoblasts at an earlier state of the process of repair.

FREMONT-SMITH: The whole question is one of timing. You agree that mitosis did take place earlier. Dr. Urist says mitosis is important for induction, and then you got bone, and all Dr. Pritchard is insisting on, quite correctly, I think, is that there was not any very active mitosis just before the bone was formed, and Dr. Urist says there was a lot of active mitosis earlier.

CURREY: Which resulted in tendon cells.

PRITCHARD: Which later resulted in bone cells. That is the point. There is an intermediary stage of maturity between the mitotic state and the bone-cell state. If the cells remained immature for several weeks, it would be different.

FREMONT-SMITH: The question is: How long can you have a delay and still call it induction? This is really what the argument is about.

URIST: Osteoblasts do not undergo mitosis. At the stage of development of osteoblasts, the induction system has reached the stage in which mitosis is not an essential feature. Before osteoblasts appear and bone induction can occur, cellular proliferation is a requirement. A supply of new cells, both from inside and outside the tendon, seems essential to account for the large number of new cells—osteoblasts, hemocytoblasts, fibroblasts, and fat cells in a deposit of bone in tendon.

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[4] It can be demonstrated in a specimen from a patient I treated for ossification of the tendo Achilles, as shown in figures 142 and 143. When bone formation occurs, many of the new trabeculae are alined parallel to the long axis of the fibers of the original tendon, but some are formed also at right angles (fig. 142). In figure 143, new cartilage and fibrocartilage at the top of the figure, new bone at the bottom of the figure around an excavation chamber filled with osteoblasts, osteoprogenitor cells, and sprouting blood vessels at the center suggest that the ossification of the tendon occurs by replacement of new tissue and new cells, not transformation of old cells of the original tendon. If an induction system is set up, the responding or induced cells, not old tendon cells, are the chief source of the new bone. If it is argued that the fibrocartilage came from old tendon cells, there are numerous paired cells to suggest that mitotic division also occurred and continues to occur for some time before and during the osteogenic reaction. Therefore, the evidence is that mitosis must be an integral part of the process of bone induction by tendon.

Hirsch and Morgan (ref. 209) reviewed the literature on the subject of ossification of tendon and pointed out that the theory of metaplasia is unnecessary because the new bone forms in the presence of proliferating fibroblasts, fibrocartilage and cartilage, the same as in endochondral ossification.

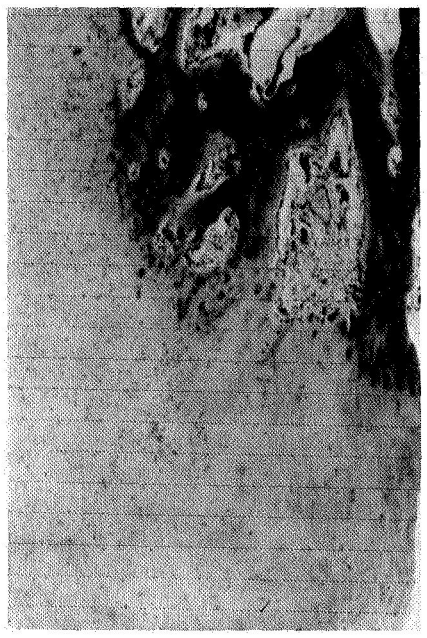

FIGURE 142. Photomicrograph of ossification area in the tendo Achilles of a 39-year-old woman. Tendon fibers and new fibrous tissue (top), new fibrocartilage-like cells (center), bone (bottom). HEA stain.

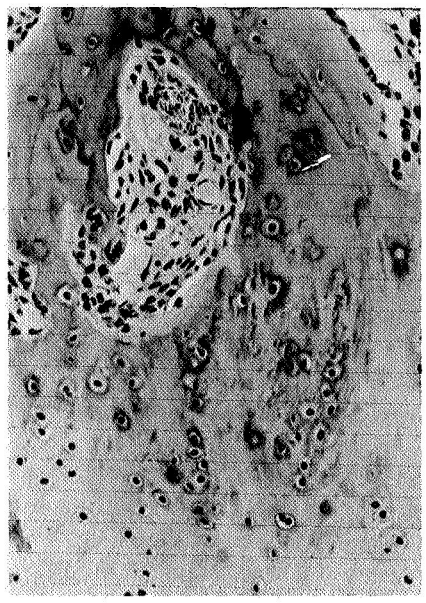

FIGURE 143. Photomicrograph showing the composition of the tissue at the line of division between tendon and bone.

NICHOLS: The question that I originally asked, which seems to have precipitated this discussion, was whether proliferation has to be actively going on at the time that the inductive act occurs? There are two possibilities: cells already formed could be "informed" to suddenly start functioning in a new way; the act of induction might only occur in tissue which was in the act of growing, that is, tissue which was ready to be plasticized. Which is it?

URIST: Dr. Holtzer, can you answer that question?

HOLTZER: There is no simple answer to your question. In part, the reason for this is the difficulty in agreeing on what induction is. The appearance of a bone cell in the developing embryo does not involve one inductive event but many, and the nature of these inductive events probably differs (ref. 210). On a molecular level, we know absolutely nothing about inductive mechanisms in metazoans. Some inductive systems may involve proteins, others lipids, others inorganic ions, others carbohydrates, and so forth.

NICHOLS: Dr. Holtzer, as I understand the "induction" we are talk-

ing about now, it is the creation of a new kind of tissue; I am asking about induction in this sense rather than in the sense you are using.

HOLTZER: That is a wonderful question. Can an exogenous molecule tell a cell that was going to make albumin to make myosin? A quick reading of the embryologic literature might, in fact, leave that impression. By appropriately manipulating bits of embryonic tissues you can demonstrate that here is a group of cells whose descendants would have formed liver cells if left *in situ.* Now, if that bit of tissue is grafted elsewhere, it is possible that the progeny of the grafted cells might differentiate into muscle. But it is worth stressing that there was no single event that can be identified as transforming presumptive liver cells into muscle cells. On the other hand, we know that there was a great deal of mitotic activity in this type of experiment.

NICHOLS: All right. But do you have to have a set of cells in your system that is already mitosing in order to be able to induce them? Or do you start with a resting cell? This is what I am trying to get at.

HOLTZER: In the induction systems I have studied, there is invariably a great deal of mitotic activity. In fact, I know of no well-studied induction system in which the cells are not replicating. Recently, we have proposed that cells might be more responsive to inductive influences immediately following mitosis. For example, we have shown that a presumptive muscle cell does not synthesize myosin until a minimum of 3 to 5 hours after a given mitosis (refs. 124 and 125). Now, for all we know, the parent cell was programed to produce daughter cells, one or both of which would synthesize myosin. Nevertheless, that particular cell division was necessary to commit the cell to making contractile proteins. Some time ago, we demonstrated that blocking mitotic activity during the inductive interaction between spinal cord and somites could, in fact, inhibit the induction of vertebral cartilage. I believe many experiments in the literature on the effects of nucleic acid inhibitors are due to their blocking cell division and not their action on RNA synthesis.[5]

Indeed, I would go much further and say that what in the past have been referred to as the stepwise events responsible for cell differentiation in the embryo are in large measure changes associated with cell divisions.

URIST: Let us leave the question of whether mitotic activity is essential for induction, and ask if it is possible to apply the hypothesis of Jacob and Monod (ref. 211) to the problem of planning some good new experiments on bone induction.

FREMONT-SMITH: Can you state the hypothesis briefly for ignorant ones like myself?

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[5] Matheson and Holtzer (unpublished observations).

URIST: The Jacob and Monod hypothesis states that the synthesis of proteins by a living organism follows a double genetic control: structural genes determine the molecular organization of the proteins; regulator and operator genes control the rate of protein synthesis through the intermediacy of cytoplasmic components or repressors. The repressors can be either inactivated (induction) or activated (repression) by certain specific metabolites. The system of regulation appears to operate directly at the level of synthesis of the gene of a short-lived intermediate, or messenger, which becomes associated with the ribosomes where protein synthesis takes place. These highly imaginative concepts were developed from a series of experiments on the synthesis of the β-galactosidase and other proteins in cultures of *Escherichia coli.* Watson (ref. 212) and other adventurous young men can be expected to attempt the application of the Jacob and Monod hypothesis to the problem of cellular differentiation and induction systems. Before it is possible to try to use the hypothesis to interpret old experiments and plan new ones on bone induction, it is advisable to define the term "induction." The definition of Jacobson (ref. 213) expresses the view of the most modern embryologists as an "interaction between one tissue [the inductor] and another responding tissue, as a result of which the responding tissue takes a course of differentiation it would not have followed had the interaction not occurred."

The appearance of the bone induction system depends upon the genetic machinery of the species. Bone induction appears only in bony vertebrates. It does not appear in the hagfish, a vertebrate that produces only connective tissue and uncalcified cartilage. It does not appear in the shark, a vertebrate that produces teeth and calcified cartilage but no bone in the skeleton. Only bony vertebrates possess connective-tissue cells with the genetic machinery to be induced to differentiate into bone cells. Later, I will demonstrate an induction system we described in an implant of decalcified bone matrix. If the Jacob and Monod (ref. 211) hypothesis were applicable to this system, it would indicate that the inducer (one or a combination of a number of factors) diffused through the cell to extend as far as the regulator gene.

HOLTZER: You really do not have to go that far, do you?

URIST: You are correct; it is not necessary for the inductor to diffuse as far into the cell as the genetic machinery, but it is also correct that it could, through the intermediacy of a cytoplasmic component or repressor.

HOLTZER: Nobody has ever talked about its hitting the gene. Why do we not leave it in the cell?

URIST: There are at least three possible targets for the inducer: the gene, the ribosomal elements, and the cell wall or plasma membrane.

SAXEN: And in all those sites it could inhibit or stimulate. So there are at least six possible targets.

MCLEAN: It is getting late and since I am now in the chair, I will adjourn this session.